(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

B5

(19) World Intellectual Property Organizati n
International Bureau

(43) International Publication Date
21 December 2000 (21.12.2000)

PCT

(10) International Publication Number
WO 00/77178 A1

(51) International Patent Classification[7]: C12N 9/00

(21) International Application Number: PCT/US00/16551

(22) International Filing Date: 15 June 2000 (15.06.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/139,408 16 June 1999 (16.06.1999) US

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(81) Designated States *(national)*: AU, CA, JP.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/77178 A1

(54) Title: IMMUNOLOGICAL CONTROL OF β-AMYLOID LEVELS *IN VIVO*

(57) Abstract: The present invention provides an antibody which catalyzes hydrolysis of β-amyloid at a predetermined amide linkage. The antibody preferentially binds a transition state analog which mimics the transition state adopted by β-amyloid during hydrolysis at a predetermined amide linkage. Specific antibodies provided include those which catalyze the hydrolysis at the amyloid linkages between residues 39 and 40, 40 and 41, and 41 and 42 of β-amyloid. The present antibody also provides a vectorized antibody which is characterized by the ability to cross the blood brain barrier and also catalyze the hydrolysis of β-amyloid. Also provided are methods for sequestering free β-amyloid in the blood stream, for reducing levels of β-amyloid in the brain, for reducing the level of circulating β-amyloid, for preventing the formation of amyloid plaques in the rain and for disaggregating amyloid plaques. Finally, this invention also provides a method of generating antibodies by immunizing an animal with antigen comprised of an epitope which has a statine analog or which utilizes reduced peptide bond analogs to mimic the conformation of a hydrolysis transition state of a polypeptide.

IMMUNOLOGICAL CONTROL OF β-AMYLOID LEVELS *IN VIVO*

Background of the Invention

Alzheimer's disease is a progressive and ultimately fatal form of dementia that affects a substantial portion of the elderly population. Definitive diagnosis at autopsy relies on the presence of neuropathological brain lesions marked by a high density of senile plaques. These extracellular deposits are found in the neo-cortex, hippocampus and amygdala as well as in the walls of the meningeal and cerebral blood vessels. The principal component of these plaques is a 39 to 43 residue β-amyloid peptide. Each plaque contains approximately 20 fmole (80 picograms) of this 4 kDa peptide (Selkoe et al., *J. of Neurochemistry* 46: 1820 (1986)). Apolipoprotein E and neurofibrillary tangles formed by the microtubule-associated tau protein are also often associated with Alzheimer's disease.

β-amyloid is proteolytically cleaved from an integral membrane protein called the β-amyloid precursor protein. The gene which codes for this protein in humans is found on chromosome 21 (St George-Hyslop et al., *Science* 235: 885 (1987), Kang et al., *Nature* 325: 733 (1987)). Numerous cultured cells and tissues (eg. brain, heart, spleen, kidney and muscle) express this β-amyloid precursor protein and also secrete the 4 kDa β-amyloid fragment into culture media, apparently as part of a normal processing pathway.

While it is difficult to establish an absolute causal relationship between β-amyloid or the plaques it forms and Alzheimer's disease, there is ample evidence to support the pathogenic role of β-amyloid. For example, patients with Down's syndrome have an extra copy of the β-amyloid precursor protein gene due to trisomy of chromosome 21 (St George-Hyslop et al., *Science* 235: 885 (1987), Kang et al., *Nature* 325: 733 (1987)). They correspondingly develop an early-onset Alzheimer's disease neuropathology at 30-40 years of age. Moreover, early-onset familial Alzheimer's disease can result from

mutations in the β-amyloid precursor protein gene which fall within or adjacent to the β-amyloid sequence (Hardy, J., *Nature Genetics* 1: 233 (1992)). These observations are consistent with the notion that deposition of β-amyloid as plaques in the brain are accelerated by an elevation in its extracellular concentration (Scheuner et al., *Nature Med.* 2: 864 (1996)). The finding that β-amyloid is directly neurotoxic both *in vitro* and *in vivo* (Kowall et al., *Proc. Natl. Acad. Sci.* 88: 7247 (1991)), suggest that soluble aggregated β-amyloid, not the plaques per se, may produce the pathology.

Observations have indicated that amyloid plaque formation may proceed by a crystallization type mechanism (Jarrett et al., *Cell* 73: 1055 (1993)). According to this model, the seed that initiates plaque nucleation is an β-amyloid which is 42 or 43 amino acids long ($A\beta_{1-43}$). The rate-determining nucleus formed by $A\beta_{1-43}$ or $A\beta_{1-42}$ allows peptides $A\beta_{1-40}$ or shorter to contribute to the rapid growth of an amyloid deposit. This nucleation phenomenon was demonstrated *in vitro* by the ability of $A\beta_{1-42}$ to cause the instantaneous aggregation of a kinetically stable, supersaturated solution of $A\beta_{1-40}$. That finding has led to the possibility that $A\beta_{1-40}$ might be relatively harmless in the absence of the nucleation peptides $A\beta_{1-42}$ or $A\beta_{1-43}$. Indeed, elevated levels of these long peptides have been found in the blood of patients with familial Alzheimer's disease (Scheuner et al., *Nature Med.* 2: 864 (1996)). Moreover, $A\beta_{1-42}$ or $A\beta_{1-43}$ was found to be the predominant form deposited in the brain plaques of many Alzheimer's disease patients (Gravina et al., *J. of Biol. Chem.* 270: 7013 (1995)).

Given the central role played by β-amyloid, it has become increasingly important to understand the interrelationship between the different pools of these molecules in the body. Free β-amyloid present in the blood most likely arises from peptide released by

proteolytic cleavage of β-amyloid precursor protein present on cells in the peripheral tissues. Likewise most of the free β-amyloid found in the brain and cerebrospinal fluid is probably derived from peptide released by secretase cleavage of β-amyloid precursor protein expressed on brain cells. The peptides are identical regardless of origin, and the results from several studies suggest an intercommunication between these pools.

Summary of the Invention

One aspect of the present invention is an antibody which catalyzes hydrolysis of β-amyloid at a predetermined amide linkage. In one embodiment, the antibody preferentially binds a transition state analog which mimics the transition state adopted by β-amyloid during hydrolysis at a predetermined amide linkage and also binds to natural β-amyloid with sufficient affinity to detect using an ELISA. In another embodiment, the antibody preferentially binds a transition state analog which mimics the transition state adopted by β-amyloid during hydrolysis at a predetermined amide linkage, and does not bind natural β-amyloid with sufficient affinity to detect using an ELISA. Antibodies generated are characterized by the amide linkage which they hydrolyze. Specific antibodies include those which catalyze the hydrolysis at the amyloid linkages between residues 39 and 40, 40 and 41, and 41 and 42, of β-amyloid.

Another aspect of the present invention is a vectorized antibody which is characterized by the ability to cross the blood brain barrier and is also characterized by the ability to catalyze the hydrolysis of β-amyloid at a predetermined amide linkage. In one embodiment, the vectorized antibody is a bispecific antibody. Preferably, the vectorized antibody has a first specificity for the transferrin receptor and a second specificity for a transition state adopted by β-

amyloid during hydrolysis. Specific vectorized antibodies include those which catalyze the hydrolysis at the amyloid linkages between residues 39 and 40, 40 and 41, and 41 and 42, of β-amyloid.

Another aspect of the present invention is a method for sequestering free β-amyloid in the bloodstream of an animal by intravenously administering antibodies specific for β-amyloid to the animal in an amount sufficient to increase retention of β-amyloid in the circulation. Therapeutic applications of this method include treating patients diagnosed with, or at risk for Alzheimer's disease.

Another aspect of the present invention is a method for sequestering free β-amyloid in the bloodstream of an animal by immunizing an animal with an antigen comprised of an epitope which is present on β-amyloid endogenous to the animal under conditions appropriate for the generation of antibodies which bind endogenous β-amyloid. Therapeutic applications of this method include treating patients diagnosed with, or at risk for Alzheimer's disease.

Another aspect of the present invention is a method for reducing levels of β-amyloid in the brain of an animal by intravenously administering antibodies specific for endogenous β-amyloid to the animal in an amount sufficient to increase retention of β-amyloid in the circulation of the animal. In one embodiment, the antibodies are catalytic antibodies which catalyze hydrolysis of β-amyloid at a predetermined amide linkage. The antibodies may be either monoclonal or polyclonal. In one embodiment, the antibodies specifically recognize epitopes on the C-terminus of β-amyloid$_{1-43}$.

Another aspect of the present invention is a method for reducing levels of β-amyloid in the brain of an animal, by immunizing the animal with an antigen comprised of an epitope which is present on endogenous β-amyloid under conditions appropriate for the generation

of antibodies which bind endogenous β-amyloid. In one embodiment, the antigen is a transition state analog which mimics the transition state adopted by β-amyloid during hydrolysis at a predetermined amide linkage. In a preferred embodiment, the antigen is comprised of $A\beta_{10-25}$. Preferably, the antibodies generated have a higher affinity for the transition state analog than for natural β-amyloid, and catalyze hydrolysis of endogenous β-amyloid.

Similar methods which utilize or generate antibodies which catalyze the hydrolysis of β-amyloid for reducing levels of circulating β-amyloid in an animal, and also for preventing the formation of amyloid plaques in the brain of an animal, are also provided. Also, methods for disaggregating amyloid plaques present in the brain of an animal by utilizing or generating antibodies which catalyze the hydrolysis of β-amyloid are provided.

Another aspect of the present invention is a method for disaggregating amyloid plaques present in the brain of an animal by intravenously administering vectorized bispecific antibodies to the animal in an amount sufficient to cause significant reduction in β-amyloid levels in the brain of the animal. The vectorized bispecific antibodies are competent to transcytose across the blood brain barrier, and have the ability to catalyze hydrolysis of endogenous β-amyloid at a predetermined amide linkage upon binding. Preferably, the vectorized bispecific antibodies specifically bind the transferrin receptor.

Another aspect of the present invention is a method for generating antibodies which catalyze hydrolysis of a protein or polypeptide by immunizing an animal with an antigen comprised of an epitope which has a statine analog which mimics the conformation of a predetermined hydrolysis transition state of the polypeptide, under conditions appropriate for the generation of antibodies to the hydrolysis transition state. This method can be

used to generate catalytic antibodies to β-amyloid. A similar method, which utilizes reduced peptide bond analogs to mimic the conformation of a hydrolysis transition state of a polypeptide, is also provided.

Brief Description of the Figures

Figure 1 is an amino acid sequence listing (SEQ ID NO: 1) of the 43 residue β-amyloid peptide (Aβ).

Figure 2 is an amino acid sequence listing (SEQ ID NO: 2) of the antigenic peptide made from the N-terminal sequence of β-amyloid ($A\beta_{1-16}$).

Figure 3 is an amino acid sequence listing (SEQ ID NO: 3) of the antigenic peptide made from the central region of β-amyloid ($A\beta_{10-25}$).

Figure 4 is an amino acid sequence listing (SEQ ID NO: 4) ($A\beta_{35-43}$) of the antigenic peptide made from the C-terminal sequence of β-amyloid.

Figure 5 is a diagrammatic representation of data from an ELISA comparing monoclonal antibody binding to $A\beta_{35-43}$ and $A\beta_{1-43}$ versus $A\beta_{1-40}$.

Figure 6 indicates the amide linkages in the peptide made from the β-amyloid C-terminal sequence (SEQ ID NO: 4) that were independently replaced with a statyl moiety, to generate the different statine transition state analogs of the peptide.

Figure 7 indicates the amide linkages in the peptide made from the β-amyloid central sequence (SEQ ID NO: 3) that were independently replaced with a statyl moiety, to generate the different phenylalanine statine transition state analogs of the peptide.

Figure 8 is a structural comparison between the native β-amyloid peptide and the transition state phenylalanine statine β-amyloid peptide analog.

Figure 9 is a structural comparison between the native β-amyloid peptide and the reduced peptide bond transition state β-amyloid peptide analog.

Figure 10 is a formulaic representation of the native C-terminal region of β-amyloid, and the phosphonamidate transition state analog of the C-terminal region of β-amyloid ($A\beta_{35-43}$).

Figure 11 indicates the putative transition state for peptide hydrolysis by zinc peptidases, compared to the phosphonate and phosphonamidate mimics.

Figure 12 is a structural comparison of the native β-amyloid peptide and the transition state phosphonamidate β-amyloid peptide which has the peptide link between Gly 38 and Val 39 replaced with a phosphonamidate bond.

Figure 13 is a diagrammatic representation of data from an ELISA which assess the binding of monoclonal antibodies generated to transition state β-amyloid peptide analogs, to the normal $A\beta_{1-43}$ and to the phenylalanine statine transition state β-amyloid peptide.

Figure 14 is a diagrammatic representation of data from an ELISA comparing antibody binding to the statine transition state β-amyloid peptide versus native $A\beta_{1-43}$ and native $A\beta_{1-40}$.

Figure 15 is a graph of data showing the cleavage of ^{125}I-Aβ-sepharose by monoclonal antibodies generated to transition state analogs of β-amyloid.

Figure 16 is a diagrammatic representation of data which quantitate the attachment of bispecific antibody to receptor-positive cells.

Figure 17 is a diagrammatic representation of data obtained from experiments designed to track the transcytosis of vectorized bispecific antibody into brain.

Detailed Description of the Invention

The present invention relates to immunologically based methods for controlling levels of β-amyloid in the body of an animal. The invention is based on the finding that antibodies specific for β-amyloid are able to bind

β-amyloid in the presence of a physiological level of human serum albumin. The invention is also based on the finding that an animal can tolerate the presence of antibodies specific for β-amyloid in amounts sufficient to sequester β-amyloid in the bloodstream.

One aspect of the present invention relates to a method for sequestering free β-amyloid in the bloodstream of an animal. The soluble and insoluble forms of β-amyloid present within an animal are in dynamic equilibrium. Soluble β-amyloid is thought to translocate between blood and cerebrospinal fluid. Insoluble β-amyloid aggregates deposit from the soluble pool in the brain, as amyloid plaques. Results detailed in the Exemplification section below indicate that intravenous administration of antibodies specific for β-amyloid to an animal impedes the passage of soluble β-amyloid out of the peripheral circulation. This occurs because the β-amyloid specific antibodies, which are restricted to the peripheral circulation, bind to β-amyloid and sequester it in the circulation. Such sequestration is accomplished through intravenous administration of an appropriate amount of antibodies specific for β-amyloid to the animal. The amount of antibody which is sufficient to produce sequestration is dependent upon various factors (e.g., specific characteristics of the antibody to be delivered, the size, metabolism, and overall health of the animal) and are to be determined on a case by case basis.

Administered antibodies can be monoclonal antibodies, a mixture of different monoclonal antibodies, polyclonal antibodies, or any combination therein. In one embodiment, the antibodies bind to the C-terminal region of β-amyloid. Such antibodies specifically bind the less abundant, but more noxious $A\beta_{1-43}$ species in the blood as opposed to the smaller and less detrimental $A\beta_{1-40}$. In another embodiment, a combination of antibodies having specificity for various regions of β-amyloid are

administered. In another embodiment, antibodies which catalyze the hydrolysis of β-amyloid, discussed in more detail below, are administered either alone or in combination with other anti-β-amyloid antibodies.

The animal to which the antibodies are administered is any animal which has circulating soluble β-amyloid. In one embodiment, the animal is a human. The human may be a healthy individual, or alternatively, may be suffering from or at risk for a disease in which elevated β-amyloid levels are thought to play a role, for example a neurodegenerative disease such as Alzheimer's disease.

A related aspect of the present invention is a method for sequestering free β-amyloid in the bloodstream of an animal by stimulating an immune response within the animal to endogenous β-amyloid. The results detailed in the Exemplification below indicate that an animal can tolerate the induction of an immune response which produces antibodies to endogenous β-amyloid, and that the presence of such antibodies will alter the distribution of β-amyloid in the body, in a similar manner as the above described method of administering β-amyloid binding antibodies. The immune response to endogenous β-amyloid is generated by immunizing the animal with one or more antigens comprised of epitopes present on the endogenous β-amyloid. Epitopes present on the inoculated antigens can correspond to epitopes present on any region of the β-amyloid molecule. In a preferred embodiment, epitopes found on the C-terminal region of β-amyloid are used to generate antibodies which specifically bind the $A\beta_{1-43}$ species as opposed to the smaller $A\beta_{1-40}$. In an alternate embodiment, a combination of different epitopes are administered to generate a variety of antibodies to β-amyloid. A more generalized immune response is generated by immunizing either with a mixture of different small peptide antigens or with the full-length 43 residue β-amyloid peptide. In another embodiment, antigens used for inoculation include transition state analogs of β-

amyloid peptides to induce antibodies which have catalytic activity directed towards β-amyloid hydrolysis, described in detail below.

The immunoreactivity of the antigens can be enhanced by a variety of methods, many of which involve coupling the antigen to an immunogenic carrier. In addition, various methods are known and available to one of skill in the art for specifically enhancing the immunogenicity of endogenous molecules or the epitopes contained therein. Various modifications can be made to the β-amyloid antigen(s) described herein to render it more compatible for human use. For example, the peptide(s), can be genetically engineered into appropriate antigenic carriers, or DNA vaccines can be designed.

The above techniques for sequestering β-amyloid in the circulation are also useful for reducing the levels of β-amyloid in the brain. Because the formation of amyloid plaques in the brain is dependent, at least in part, on the levels of free β-amyloid present in the brain, reducing brain β-amyloid levels of an animal will, in turn, reduce the formation of amyloid plaques in the brain. Therefore, the above techniques are useful for preventing the formation of amyloid plaques in the brain of an animal. This is especially applicable to an animal which is considered at risk for the development of amyloid plaques; a risk which may result from a genetic predisposition or from environmental factors. Administration of antibodies, or immunization of the animal to produce endogenous antibodies, to β-amyloid can be of therapeutic benefit to such an animal (e.g., a human who has a family history of Alzheimer's disease, or who is diagnosed with the disease).

Another aspect of the present invention relates to antibodies which are characterized by the ability to catalyze the hydrolysis of β-amyloid at a predetermined amide linkage. Experiments detailed in the Exemplification section demonstrate the generation of

different antibodies which have proteolytic activity towards β-amyloid. Such antibodies are generated by immunizing an animal with an antigen which is a transition state analog of the β-amyloid peptide. A transition state analog mimics the transition state that β-amyloid adopts during hydrolysis of a predetermined amide linkage. Transition state analogs useful for generating the catalytic antibodies include, without limitation, statine, phenylalanine statine, phosphonate, phosphonamidate, and reduced peptide bond transition state analogs.

Antibodies generated to epitopes unique to the transition state preferentially bind β-amyloid in the transition state. Binding of these antibodies stabilizes the transition state, which leads to hydrolysis of the corresponding amide bond. The particular amide linkage to be hydrolyzed is chosen based upon the desired cleavage product. For example, cleavage of full length β-amyloid into two peptide fragments which cannot aggregate into amyloid plaques would be of therapeutic use in the methods disclosed herein. Antibodies may be either monoclonal or polyclonal. Several such transition state mimics have been made and used as antigen in the generation of monoclonal antibodies which catalyze the cleavage at the indicated linkage. These antigens and the antibodies generated are listed in Table 8 of the Exemplification section below. Antibodies generated to antigens which have transition state mimics incorporated at a specific amide linkage, should bind the natural hydrolysis transition states of these linkages in native β-amyloid, stabilizing the transition state and catalyzing cleavage at that linkage.

At least two different classes of antibodies are generated by the above methods. The first class preferentially binds the transition state analog, and also detectably cross reacts with natural β-amyloid using the ELISA detailed in the Exemplification section, to

detect binding. The second class binds the transition state analog, and does not detectably cross react with natural β-amyloid using the ELISA procedure detailed in the Exemplification section to detect binding. Both classes of antibodies have potential value as catalytic antibodies. The respective binding affinities of an anti-transition state antibody is likely to reflect its activity at catalyzing hydrolysis. It is thought that in order for an antibody to have activity at catalyzing hydrolysis of a protein, it must possess at least a minimal ability to bind the natural (non-transition) state of the protein. Antibodies which retain significant binding for β-amyloid, (that strongly cross react with natural β-amyloid) may be more efficient at catalyzing hydrolysis due to a higher efficiency of binding the β-amyloid. Once bound, these antibodies force the protein into a transition state conformation for hydrolytic cleavage. Alternatively, antibodies which only minimally cross react with natural β-amyloid, although less efficient at binding native β-amyloid, are likely to be more efficient at forcing the bound β-amyloid into the transition state conformation for hydrolytic cleavage. It should be pointed out that failure to detect binding of the anti-transition state antibodies to natural β-amyloid by the ELISA methods presented in the Exemplification herein does not necessarily reflect an inability to bind natural β-amyloid sufficiently to function as a catalytic antibody. More likely, a lack of detection merely reflects the sensitivity limitations of the assay.

Antibodies which have substantial affinity for the predicted cleavage products of the native β-amyloid peptide may be subject to product inhibition and might therefore exhibit low turnover. Such undesirable antibodies can be identified by secondary screening using peptides which contain epitopes of the predicted cleavage products (e.g., via ELISA).

In a preferred embodiment, the antibodies are monoclonal. Monoclonal antibodies are produced by immunizing an animal (e.g., mouse, guinea pig, or rat) with the transition state analog antigen, and subsequently producing hybridomas from the animal, by standard procedures. Hybridomas which produce the desired monoclonal antibodies are identified by screening. One example of a screening method is presented in the Exemplification section which follows. In another embodiment, the antibodies are polyclonal. Polyclonal antibodies are generated by immunizing an animal (e.g., a rabbit, chicken, or goat) with antigen and obtaining sera from the animal. Polyclonal antibodies which have the desired binding specificities can be further purified from the sera by one of skill in the art through the course of routine experimentation.

Catalytic antibodies specific for β-amyloid can alternatively be generated in an individual through the use of anti-idiotype vaccines designed to elicit the production of catalytic antibodies. Such vaccines are described in the disclosure of Raso and Paulus (U.S. Patent Application 09/102,451, ANTI-IDIOTYPE VACCINES TO ELICIT CATALYTIC ANTIBODIES, filed by Applicants June 22, 1998, currently pending), the contents of which are incorporated herein by reference.

Another aspect of the present invention is the use of statine and reduced peptide bond analogs to elicit catalytic antibodies having proteolytic activity. The Exemplification section below details methods for using statine analogs as antigen in the production of catalytic antibodies, and also lists examples of anti-transition-state antibodies generated using these methods. The "statyl" moiety is derived from naturally evolved protease transition state inhibitors like amastatin, pepstatin, and bestatin. These naturally-occurring statine-based inhibitors have been used to effectively block the activity of aminopeptidases, aspartic proteases

and the HIV protease. Synthetic peptides containing a statine residue offer novel features for the induction of catalytic antibodies. The statyl moiety has a tetrahedral bond geometry, its length is extended by two CH_2 units, it has a strategically placed OH group and the structure has no charge. The presence of the additional CH_2 units is expected to elicit a more elongated antibody combining site, and antibodies possessing this extended site will induce extra strain on the peptide substrate, producing an accelerated catalysis. In addition, the -OH group in these statine analogs is thought to better approximate the position and chemistry of the true transition state. Statine-based transition-state analogs should therefore elicit a class of antibodies which is significantly different from those obtained from the more commonly used negatively charged phosphonate analogs.

Reduced peptide bond analogs introduce a tetrahedral configuration, without increasing the distance between amino acid residues. This feature should more closely approximate the true transition state geometry, than previously used analogs. A positively charged secondary amine replaces the amide nitrogen of the natural polypeptide and should elicit a complementary negatively charged side chain at a proximal locus in the antibody combining site. The presence of such ancillary glutamyl or aspartyl groups on the antibody will assist antibody-mediated catalysis of peptide cleavage via acid-base exchange. Reduced peptide bond-based transition-state analogs should therefore elicit a class of antibodies which is significantly different from those obtained from using the more commonly used negatively charged phosphonate analogs. Reduced peptide bond analogs and statine analogs can be used to produce specific transition state analog antigens for a wide variety of proteins or polypeptides. These antigens can in turn be used to generate the respective catalytic antibodies.

Administration of the β-amyloid catalytic antibodies described above can be used in the methods described above for 1) sequestering free β-amyloid in the bloodstream of an animal, 2) reducing levels of β-amyloid in the brain of an animal, and 3) preventing the formation of amyloid plaques in the brain of an animal, to generate the analogous results. Experiments presented in the Exemplification demonstrate that immunization of an animal with a transition state analog results in the generation of an immune response to produce antibodies which recognize the transition state, and which catalyze hydrolysis of the β-amyloid protein. This indicates that the transition state analogs can be used as antigens in these methods to induce the production of antibodies in the animal which recognize and catalyze cleavage of endogenous β-amyloid.

Methods which involve reducing overall levels of β-amyloid in an animal through the proteolytic action of the above described catalytic antibodies are also encompassed by the present invention. The presence of functional catalytic antibodies in the circulation of an animal will reduce the level of intact β-amyloid in the circulation by selective hydrolytic cleavage. Accordingly, the present invention provides a method for reducing levels of circulating β-amyloid in an animal by introducing the above described catalytic antibodies into the animal. Administration of the antibodies to the animal is preferably via intravenous administration. Such antibodies are either monoclonal, mixed monoclonal, polyclonal or any mixture thereof. The origin of the antibody may affect the half-life of the antibody in the animal; antibodies from less related species are more likely to be recognized as foreign by the animal's immune system. Preferably, administered antibodies are derived from a species closely related to the animal, to maximize half-life and minimize adverse reactions by the host.

Administration of isolated variable region antibody fragments may produce beneficial results in this regard.

The present invention also provides a method for reducing levels of circulating β-amyloid in an animal by immunizing the animal with a β-amyloid transition state analog to induce endogenous catalytic antibody production. The use and design of such vaccines is described above, and detailed in the Exemplification section below.

The reduction of β-amyloid levels in the circulation of an animal is expected to displace the equilibrium of β-amyloid in the body, and ultimately lead to a reduction in the levels of β-amyloid in the brain of the animal through mass action. In this respect, the present invention provides methods for reducing the levels of β-amyloid in the brain of an animal, by either administering catalytic antibodies to the animal, or by administering a transition state analog to induce endogenous antibody production. It follows that these procedures also have value as methods for preventing the formation of amyloid plaques in the brain of an animal, since the resulting reduction in the levels of β-amyloid in the brain of an animal should prevent the formation of amyloid plaques. These procedures also have value as methods for disaggregating amyloid plaques present in the brain of an animal, since evidence indicates that lower brain β-amyloid levels can lead to the disaggregation of plaques.

Another aspect of the present invention provides a more direct method of altering the distribution of β-amyloid in the brain by actually delivering anti-β-amyloid antibodies to the brain. Methods described above for reducing levels of β-amyloid in the brain and for preventing aggregation of amyloid plaques depend upon exchange between β-amyloid pools in the blood, tissues, cerebrospinal fluid and the brain, the exchange being driven by an antibody-mediated disruption of the

equilibrium between these different pools. In contrast, delivery of anti-β-amyloid antibodies to the brain will directly affect β-amyloid aggregation. Evidence presented in the Exemplification section below indicates that the binding of certain anti-β-amyloid antibodies inhibits the initial aggregation of β-amyloid *in vitro*, and also disaggregates preformed *in vitro* β-amyloid complexes. Moreover, if insoluble peptide is in equilibrium with a low level of soluble β-amyloid, then an anti-β-amyloid binding antibody could upset this balance and gradually dissolve the precipitate. These observations indicate that the presence of β-amyloid antibodies in the brain will directly inhibit the formation of amyloid plaques and will also disaggregate preformed plaques by disrupting the dynamic equilibrium between soluble β-amyloid and fibrillar β-amyloid deposited as plaques. Furthermore, a highly active catalytic antibody is expected to destroy insoluble β-amyloid plaques by hydrolytically cleaving the constituent aggregated peptides.

One way of delivering antibodies to the brain is by producing vectorized antibodies competent for transcytosis across the blood-brain barrier. Vectorized antibodies are produced by covalently linking an antibody to an agent which promotes delivery from the circulation to a predetermined destination in the body. Examples of vectorized molecules which can traverse the blood-brain barrier are found in the prior art (Bickel et al., *Proc. Natl. Acad. Sci. USA* 90: 2618-2622 (1993); Broadwell et al., *Exp. Neurol.* 142: 47-65 (1996)). In these examples, antibodies are linked to another macromolecule, the antibodies being the agent which promotes delivery of the macromolecules. One example of such an agent is an antibody which is directed towards a cell surface component, such as a receptor, which is transported away from the cell surface. Examples of antibodies which confer the ability to trancytose the blood-brain barrier

include, without limitation, anti-insulin receptor antibodies, and also anti-transferrin receptors (Saito et al., *Proc. Natl. Acad. Sci. USA* 92: 10227-31 (1995); Pardridge et al., *Pharm. Res.* 12: 807-816 (1995); Broadwell et al., *Exp. Neurol.* 142: 47-65 (1996)). This first antibody is covalently linked to an antibody which binds β-amyloid. Alternatively, coupling the β-amyloid antibodies to ligands which bind these receptors (e.g., insulin, transferrin, or low density lipoprotein) will also produce a vectorized antibody competent for delivery to the brain from the circulation (Descamps et al., *Am. J. Physiol.* 270: H1149-H1158 (1996); Duffy et al., *Brain Res.* 420: 32-38 (1987); Dehouck et al., *J. Cell Biol.* 138: 877-889 (1997)).

A vector moiety can be chemically attached to the anti-β-amyloid antibody to facilitate its delivery into the central nervous system. Alternatively, the moiety can be genetically engineered into the antibody as an integral component. This vector component can be for example, an anti-transferrin receptor antibody or anti-insulin receptor antibody which binds the receptors present on the brain capillary endothelial cells (Bickel et al., *Proc. Natl. Acad. Sci. USA* 90: 2618-22 (1993); Pardridge et al., *J. Pharmacol. Exp. Ther.* 259: 66-70 (1991); Saito et al., *Proc. Natl. Acad. Sci. USA* 92: 10227-31(1995); Friden et al., *J. Pharm. Exper. Ther.* 278: 1491-1498 (1996)) which make up the blood-brain barrier. The resulting bifunctional antibody will attach to the appropriate receptors on the luminal side of the vessel (Raso et al., *J. Biol. Chem.* 272: 27623-27628 (1997); Raso et al., *J. Biol. Chem.* 272: 27618-27622 (1997); Raso, V. *Anal. Biochem.* 222: 297-304 (1994); Raso et al., *Cancer Res.* 41: 2073-2078 (1981); Raso et al., Monoclonal antibodies as cell targeted carriers of covalently and non-covalently attached toxins. *In* Receptor mediated targeting of drugs, vol. 82. G. Gregoriadis, G. Post, J. Senior and A. Trouet, editors.

NATO Advanced Studies Inst., New York. 119-138 (1984)). Once bound to the receptor, both components of the bispecific antibody pass across the blood-brain barrier by the process of transcytosis. Anti-β-amyloid antibodies which have entered the brain interact directly with both β-amyloid plaques and the soluble β-amyloid pool. It has been estimated that concentrations of macromolecules in the 10^{-8}-10^{-7} M range can be achieved in the brain using vector-mediated delivery via these brain capillary enriched protein target sites (Maness et al., *Life Sciences* 55: 1643-1650 (1994); Lerner et al., *Science* 252: 659-667 (1991)). Importantly, the vector appears safe since animals dosed daily for two weeks with an anti-transferrin receptor antibody displayed no loss of integrity of the blood-brain barrier, using a radioactive sucrose probe (Broadwell et al., *Exp. Neurol.* 142: 47-65 (1996)).

The Exemplification details the production of vectorized bispecific antibodies which bind β-amyloid. The bispecific antibodies transcytose across the blood brain barrier via a first specificity which binds the transferrin receptor. Use of antibodies which bind the transferrin receptor for delivery of agents across the blood brain barrier is described by Friden et al. in U.S. Patent No. 5,182,107; No. 5,154,924; No. 5,833,988; and No. 5,527,527; the contents of which are incorporated herein by reference.

Results from experiments presented in the Exemplification section which follows indicate that the produced bispecific antibodies retain their separate specificities and are delivered across the blood-brain barrier into the brain parenchyma and brain capillaries of a live animal when administered intravenously.

Alternate methods for the production of bispecific antibodies have been described for genetically engineering bispecific reagents or for producing them intracellularly by fusing the two different hybridoma

clones (Holliger et al., *Proc. Natl. Acad. Sci.* 90: 6444-6448 (1993); Milstein et al., *Nature* 305: 537 (1983); Mallander et al., *J. Biol. Chem.* 269: 199-206 (1994)). Vectorized bispecific antibodies produced by these techniques can also be used in the methods of the present invention.

Since the introduction of whole antibodies into the brain might be detrimental if they were to fix complement and promote complement-mediated lysis of neuronal cells, it may be beneficial to produce and utilize smaller vectorized $F(ab')_2$ bispecific reagents. It has been shown that aggregated β-amyloid itself can fix complement in the absence of any antibody and that the resulting inflammation may contribute to the pathology of Alzheimer's disease. The possibility of intracerebral antibody having a similar effect can be greatly reduced by eliminating the Fc region of the antibody. Moreover, since coupling of Fab' halves uses the intrinsic hinge region cysteines, no extraneous substituent linkage groups need be added. Faster or more efficient entry into the brain represents another potential advantage that smaller $F(ab')_2$ or Fv_2 reagents may provide for intracerebral delivery. In addition, the two types of vectorized molecules may have different biodistribution and plasma half-life characteristics (Spiegelberg et al., *J. Exp. Med.* 121: 323 (1965)).

Depending on their design, anti-β-amyloid bispecific antibodies in the brain can reduce soluble β-amyloid and β-amyloid deposits by three potential mechanisms. An anti-β-amyloid bispecific antibody that tightly binds soluble β-amyloid will not only sequester the peptide but, due to efflux of vectorized molecules from the central nervous system (Kang et al., *J. Pharm. Exp. Ther.* 269: 344-350 (1994)), may also carry the bound β-amyloid out of the brain, releasing it into the blood stream. Such a clearance mechanism would lead to a continuous cycling of β-amyloid out of the brain. In addition, if

the antibodies have catalytic activity, they will directly reduce the levels of harmful β-amyloid by degradation. Since catalytic antibodies exhibit turnover, each antibody can inactivate many β-amyloid molecules. Thus much less vectorized bispecific antibody has to be delivered into the brain to achieve the desired depletion of β-amyloid.

To be effective the anti-β-amyloid sites of a bispecific antibody must be empty before passage out of the blood and into the brain. Therefore the concentration of bispecific antibody in animals must exceed the level of β-amyloid circulating in the blood. Calculations performed based upon known β-amyloid levels (Scheuner et al., *Nature Med.* 2: 864-870 (1996)) and a medium-range plasma level of bispecific antibody expected in a treated animal indicated 99.9% of the bispecific antibodies that enter the brain will have unoccupied anti-β-amyloid combining sites.

Another way of delivering antibodies to the brain is via direct infusion of anti-β-amyloid antibodies into the brain of an animal. This technique gives these antibodies immediate access to β-amyloid in the brain without having to cross the blood-brain barrier. Direct infusion can be accomplished via direct parenchymal or intracerebroventricular infusion (Knopf et al., *J. Immunol.* 161: 692-701 (1998)). Briefly, the animal is anesthetized and placed in a stereotaxic frame. A midsagittal incision is made on the scalp to expose the skull and the underlying fascia is scraped away. A hole is drilled to accept a sterilized length of stainless steel hypodermic tubing, which is stereotaxically advanced so that its tip is appropriately located in the brain. A guide cannula is then attached to the skull and sealed. The cannula remains in place for multiple infusions of antibody into the brain. A bolus of a sterile 50 mg/ml solution of a monoclonal anti-β-amyloid

can be infused over a 2-8 minute period into an immobilized animal via an injection cannula.

Delivery of catalytic antibodies into the brain of an animal via one of the above described methods, can also be used to disaggregate amyloid plaques present in the brain. The advantage of delivering an β-amyloid-specific catalytic antibody into the brain is two-fold. The β-amyloid peptide is permanently destroyed by such antibodies and, since catalysis is continuous, each antibody inactivates many target β-amyloid molecules in the brain. Thus much less antibody has to be infused into the central nervous system to achieve the desired depletion of β-amyloid.

The amount of antibody to be administered or delivered to the animal should be sufficient to cause a significant reduction in β-amyloid levels in the brain of the animal. The appropriate amount will depend upon various parameters (e.g., the particular antibody used, the size and metabolism of the animal, and the levels of endogenous β-amyloid) and is to be determined on a case by case basis. Such determination is within the means of one of average skill in the art through no more than routine experimentation.

It is expected that additional benefits with respect to lowering brain β-amyloid levels and preventing or disaggregating amyloid plaques can be achieved through utilizing a combination of one or more of the above described approaches.

Exemplification

SECTION 1: RETENTION OF β-AMYLOID IN THE CIRCULATION

Synthesis of β-Amyloid Peptide Antigens

The amino acid sequence of the 43 residue β-amyloid peptide (Aβ) is listed in Figure 1. To determine which sites on this Aβ peptide were best suited for antibody-

mediated therapy, three key regions (amino-terminal, central and carboxy-terminal) of the Aβ 43-mer were chosen to generate epitope-specific vaccines. These shortened peptides served as antigenic epitopes to induce a highly specific antibody response.

Monoclonal antibodies to the amino-terminal region of Aβ have been shown in the past to have the ability to solubilize Aβ aggregates (Solomon et al., *Proc. Natl. Acad. Sci. USA* 94(8): 4109 (1997); Solomon et al., *Proc. Natl. Acad. Sci. USA* 93(1): 452 (1996)). A peptide consisting of the amino-terminal region of Aβ was similarly designed for the present experiments (shown in Fig. 2 and listed in SEQ ID NO: 2) and used to elicit amino-terminal specific antibodies that bind Aβ. A Cys residue was added to the C-terminus of the Aβ sequence to provide a suitable linkage group for coupling this peptide to an antigenic carrier protein such as maleimide-activated Keyhole Limpet Hemocyanin (KLH).

A peptide encompassing the central region of Aβ was also synthesized (shown in Figure 3 and listed in SEQ ID NO: 3). A Cys residue was placed at the N-terminus of the Aβ sequence to provide a sulfhydryl linkage group for coupling the peptide to antigenic carrier proteins such as maleimide-activated KLH.

To produce an antigen for eliciting an immune response directed against the carboxy-terminus of Aβ (Suzuki et al., *Science* 264: 1336 (1994)), a decapeptide encompassing the N-terminal region of Aβ, with an additional Cys residue at the N-terminus, was synthesized (Shown in Fig. 4, and listed in SEQ ID NO: 4). The Cys substitution was designed to provide a sulfhydryl linkage group for coupling the peptide to antigenic carrier proteins such as KLH.

Coupling the Peptides to an Antigenic Carrier Protein

The different Cys containing Aβ peptides were individually thioether-linked to maleimide-activated KLH.

A multivalent Aβ vaccine was also produced by simultaneously linking all three of these peptides to maleimide-activated KLH. In addition the full-length Aβ 43-mer was linked to KLH using glutaraldehyde.

Antibodies Elicited with the β-Amyloid Vaccines

Normal BALB/c mice were immunized by standard procedures with the KLH-linked Aβ vaccines described above. The mice were either bled or sacrificed for removal of the spleen for hybridoma production. Sera and monoclonal antibodies obtained were characterized for binding to Aβ.

Table 1 shows the results from an ELISA run with 1/100 diluted serum from two non-immunized control mice versus 1/100 and 1/1000 diluted serum from a mouse that was immunized with a central region Aβ peptide-KLH vaccine. The free Aβ peptide was adsorbed directly onto the microtitre plate to avoid detection of anti-KLH antibodies in the serum.

Table 1 ELISA for Binding to the Central Region Aβ Peptide

Bound Addition		Antibody (O.D. 450 nm)
Control Serum A	1/100	0.666
Control Serum B	1/100	0.527
Mouse 1 antiserum	1/100	3.465
Mouse 1 antiserum	1/1000	2.764

Monoclonal antibodies raised against this central region Aβ peptide and produced by hybridoma fusion were identified using the above described ELISA assay. A binding assay was performed to determine whether the

monoclonal anti-Aβ antibodies identified also bound to the full length Aβ peptides. ^{125}I-Aβ$_{1-43}$ probe was incubated with hybridoma secretions from the indicated clones. A standard polyethylene glycol separation method was used to detect ^{125}I-Aβ$_{1-43}$ bound antibody (Table 2). Results presented in Table 2 indicate that the antibodies generated to the peptide fragments also bound full length Aβ$_{1-43}$.

Table 2 ^{125}I-Aβ$_{1-43}$ Binding Assay

Addition		^{125}I-Aβ$_{1-43}$ Bound (cpm)
Control Hy		3,171
Control Hy		2,903
6E2		15,938
6E2	1/10	9,379
3B1		12,078
3B1	1/10	3,353
8E3		10,789
8E3	1/10	3,249

It was reported that when ^{125}I-Aβ$_{1-40}$ is added to human plasma, ~89% binds to albumin (Biere et al., *J. of Biol. Chem.* 271(51): 32916 (1996)). This raises the concern that the bound albumin will interfere with antibody binding. Binding assays were performed in the presence and absence of serum albumin, to determine whether albumin binding interferes with antibody binding to Aβ. The ability of purified 5A11 monoclonal anti-Aβ antibody to bind ^{125}I-Aβ$_{1-40}$ was unaffected by the presence of human serum albumin (HSA) at 60 mg/ml, even though this was a 500-fold molar excess over the antibody concentration

(Table 3). These results indicate that the ability of antibodies to bind to and sequester Aβ in the blood will not be attenuated by the presence of other binding proteins.

Table 3. ^{125}I-Aβ$_{1-40}$ Binding to Antibody in the Presence of Human Serum Albumin*

Addition	^{125}I-Aβ$_{1-40}$ Bound (cpm)	Specifically Bound (% of total added)
Control	8,560	-
+ 5A11 anti-Aβ	64,589	79
Control + HSA*	3,102	-
+ 5A11 anti-Aβ + HSA*	55,304	75

*HSA at 60 mg/ml (~1 mM); anti-Aβ 5A11 at $2x10^{-6}$ M; Added ~70,000 cpm of ^{125}I-Aβ$_{1-40}$

Monoclonal Antibody Production

A mouse was immunized with a KLH conjugate of the central region Aβ$_{10-25}$ peptide (This peptide antigen had a phenylalanine statine transition state analog at an amide linkage, discussed further in Section II, below). A hybridoma fusion was performed and the resulting monoclonal antibodies analyzed to characterize the specificity of the immune response to the vaccine. Hybridoma supernatants produced in the fusion were screened using ELISA to assess their binding to the Aβ$_{1-43}$ peptide.

The monoclonal antibodies produced were determined to bind to the Aβ$_{1-43}$ peptide adsorbed directly onto an ELISA plate. Strong color reactions were obtained in this ELISA using only 10 μl of hybridoma supernatant while the addition of media alone produced low background color. These results indicate that the antibodies not

only bound to the small peptide immunogen but they were also reactive with the full-length $A\beta_{1-43}$. Importantly, antibodies bound to the carrier-free Aβ peptide adsorbed directly onto microtitre plates, showing their specificity for the peptide rather than the immunogenic carrier. The high affinity 5A11 monoclonal antibody (Table 3) was obtained from this hybridoma fusion.

A second mouse was immunized with a KLH conjugate of the $A\beta_{35-43}$ analog encompassing the C-terminal region of Aβ. Serum from the mouse was screened for reaction with $A\beta_{1-43}$ adsorbed directly onto the ELISA wells. The assay results are presented in Table 4. The spleen of this mouse was then used for a hybridoma fusion to further characterize the specificity of its immune response. Importantly, none of the mice immunized with Aβ vaccines or the anti-Aβ ascites-producing mice displayed ill effects even though some of those induced antibodies cross-react with mouse Aβ and mouse amyloid precursor protein.

Table 4 ELISA for Binding of Antiserum Directed to the Carboxy-terminal Aβ Peptide

	Antibody Bound (O.D. 450 nm)
Addition	Native $A\beta_{1-43}$
Control Serum	0.484
Mouse Antiserum	1.765

Monoclonal antibodies from hybridoma clones generated above were screened for binding to the small carboxy-terminal peptide $A\beta_{35-43}$ and the full-length $A\beta_{1-43}$. Results are presented in Figure 5. The monoclonal antibodies bound to the carboxy-terminal locus on each of these carrier-free Aβ peptides adsorbed directly to the microtitre plate, confirming their specificity for the peptide rather than the immunogenic carrier. The clones

were also tested with $A\beta_{1-40}$ to identify antibodies which do not react with this shortened, 40 amino acid residue version of $A\beta$ and thus will specifically bind to the carboxy-terminus of $A\beta_{1-43}$ (Fig. 5). Used therapeutically, this vaccine should elicit antibodies which will preferentially bind the less abundant, but more noxious $A\beta_{1-43}$ species in the blood as opposed to the smaller and less detrimental $A\beta_{1-40}$.

In a separate experiment, mice were immunized with a vaccine comprised of a cocktail of the three distinct KLH-peptide antigens (Figs. 2-4) representing the distinct regions of β-amyloid (Fig. 1). Control mice were immunized with KLH alone. The antigens were emulsified in complete Freunds adjuvant prior to the first injection and in incomplete Freunds adjuvant for subsequent injections. Tests were performed on diluted serum from these $A\beta$-KLH immunized mice to determine the presence of specific anti-$A\beta$ antibodies. The $A\beta_{1-16}$, $A\beta_{14-25}$, $A\beta_{34-43}$, $A\beta_{1-40}$, and $A\beta_{1-43}$ peptides were used to identify antibody specificity. The peptides were adsorbed directly onto an ELISA plate. The results are presented in Table 5. The results indicate that mice immunized with the cocktail of the three peptide antigens produced serum containing antibodies which react with the amino-terminal, central region, and carboxyl-terminal peptides, as well as with the full-length $A\beta$ 40-mer and 43-mer. The constant presence of this spectrum of anti-$A\beta$ antibodies will be very effective in binding all of the soluble $A\beta$ in the peripheral circulation of a vaccinated animal.

Table 5 ELISA to Measure the Serum Antibodies Present in Immunized Mice

	ELISA READING (O.D. 450 nm)				
Immunogen	$A\beta_{1-16}$	$A\beta_{14-25}$	$A\beta_{34-43}$	$A\beta_{1-40}$	$A\beta_{1-43}$
Mouse 1 (Control) KLH	0.076	0.038	0.064	0.042	0.066
Mouse 2 Aβ-KLH Cocktail	3.013	1.258	3.191	2.337	2.598
Mouse 3 Aβ-KLH Cocktail	1.484	1.180	2.068	1.758	1.680
Mouse 4 Aβ-KLH Cocktail	1.486	1.072	2.276	1.444	1.709

Vaccine Trials in Non-human Primates

Given the potential importance of β-amyloid vaccine therapy for human patients of Alzheimer's disease, a human-compatible, alum-based Aβ peptide vaccine preparation has been tested in non-human primates. Antibody production and safety studies for the human-compatible β-amyloid vaccines have commenced in Cynomolgus monkeys (*Macaca fascicularis*). This animal system is highly relevant to human applications since the predicted amino acid sequence of β-amyloid in these primates is identical to humans, and their basic physiology and immunological systems closely approximate those which will be encountered in a clinical situation. Cynomolgus monkeys were vaccinated monthly and were periodically bled to monitor anti-Aβ levels in the serum. The monkeys were also observed for any ill effects.

The Cynomolgus monkeys mounted a strong immune response to a single injection of the simplest vaccine preparation composed of the full length β-amyloid peptide adsorbed to an aluminum hydroxide gel. The specificity of those early anti-β-amyloid antibodies was characterized by ELISA using various Aβ peptide fragments (Table 6). This analysis indicated that the monkeys produced antibodies that bind to the full-length peptide and react with its amino-terminal, central and carboxyl-terminal regions.

Table 6 ELISA to Measure the Serum Antibodies Present in Aβ Vaccinated *Macaca fascicularis*

	ELISA READING (O.D. 450 nm)				
Vaccination Schedule	$A\beta_{1-16}$	$A\beta_{14-25}$	$A\beta_{33-43}$	$A\beta_{1-40}$	$A\beta_{1-43}$
Pre-Vaccination	0.511	0.404	0.370	0.380	0.235
Aβ/Alum (1st month)	2.115	1.687	0.671	2.393	2.479

Importantly, the vaccinated monkeys are perfectly healthy and appear compatible with the anti-Aβ antibodies that have been circulating in their body for over three months. Thus far, there are no apparent side effects due to cross-reaction of the anti-Aβ antibodies with naturally occurring β-amyloid precursor protein or other vital components. These animals were closely observed by a veterinarian, and have exhibited no signs of autoimmune disease, immune complex disease or any other adverse/toxic reaction to the vaccination.

In continuing experiments boost injections will be performed as per usual methods. The sera produced will be monitored for antibody specificity and affinity parameters as the immune response intensifies and matures. At termination, a complete necropsy and histopathological examination will be performed on the monkeys. Genetically engineered Aβ vaccines, discussed below, will also be evaluated in the Cynomolgus monkeys to determine if they will prove to be even better immunogens.

Antibodies Affect the Distribution of ^{125}I-Aβ in Normal Mice

Anti-Aβ antibodies in the circulation cannot cross the blood-brain barrier to a significant extent and therefore should act as a sink that prevents ^{125}I-$A\beta_{1-40}$ from reaching the brain. This retention effect was demonstrated by measuring the blood levels in mice 4 h after injecting them with equal amounts of ^{125}I-$A\beta_{1-40}$

either alone or along with our 5A11 anti-Aβ monoclonal antibody (Table 7). The passage of ^{125}I-Aβ$_{1-40}$ out of the peripheral circulation was greatly curtailed in animals which concomitantly received the specific anti-Aβ antibody. That finding extends the *in vitro* results obtained with the 5A11 antibody (Table 3) by demonstrating the antibody can effectively bind Aβ in an experimental animal. The observation that animals treated with this antibody retained 10-times more ^{125}I-Aβ$_{1-40}$ in the circulation indicates that the equilibrium distribution of Aβ in the body can be dramatically altered by selective sequestration in the blood.

Table 7 Anti-Aβ Antibody Impedes the Passage of ^{125}I-Aβ$_{1-40}$ Out of the Circulation

Mouse Injected With;	^{125}I-Aβ$_{1-40}$ in Blood (cpm/gm)
^{125}I-Aβ$_{1-40}$ alone	27,300
^{125}I-Aβ$_{1-40}$ + 5A11 anti-Aβ	278,900

Genetically Engineered Vaccines

Genetically engineered β-amyloid antigen vaccines for use in humans are currently being developed in order to induce protective levels of anti-β-amyloid antibodies. β-amyloid fragments will be engineered into chimeric Aβ vaccines which incorporate highly immunogenic carrier moieties to increase the appropriate antigenic response in a human patient. Carrier moieties suitable for use include diphtheria toxoid (DT) and the hepatitis B core antigen (HBcAg). These represent powerful delivery systems for β-amyloid peptides, and are known to induce an excellent, high titer immune response when used with alum as an adjuvant.

DT is licensed for use as a conjugate vaccine for *H. influenzae* type B and renders this immunogen T-cell dependent. The expression of DT in recombinant *E. coli*

is high. One or more of the above described β-amyloid peptides will be fused at the C-terminus of the catalytic domain of DT, or at either end of the combined transmembrane/receptor binding domains of DT. The produced fusions will be used with an aluminum hydroxide gel adjuvant to generate potent vaccines.

High titers of antibody directed against heterologous epitopes have been produced using the HBcAg delivery systems and aluminum hydroxide gel adjuvant. HBcAg has several distinct advantages as a fusion partner for Aβ peptides. The immunodominant internal site between amino acids 75 and 81 can accommodate heterologous sequences up to 45 amino acids. The core self-assembles into larger 27nm particles that are highly immunogenic. Furthermore, HBcAg can be produced in recombinant *E. coli* at elevated levels.

The genetically engineered vaccines produced will be tested for effectiveness in depleting or preventing plaques using mouse and other relevant animal models. Antibody production and safety trials for the vaccines will be conducted in Cynomolgus monkeys.

Methods of the Invention

Peptide synthesis. The 40mer $A\beta_{1-40}$, the 43mer $A\beta_{1-43}$, and the three small Aβ peptides $A\beta_{1-16}$, $A\beta_{10-25}$, and $A\beta_{35-43}$, were synthesized by standard automated Fmoc chemistry. Newly synthesized peptides were purified by HPLC and their composition was verified by mass spectral and amino acid analysis. The Aβ 43mer was obtained from a commercial source (Bachem, Torrance, CA).

Conjugation of β-Amyloid Peptides to Immunogenic Carriers. The small Aβ peptides were linked to the KLH carrier protein in order to render them antigenic. A Cys residue was strategically placed at the N- or C-terminal end of these Aβ peptides to provide a suitable linkage

group for coupling them via a thioether bond to maleimide activated carrier proteins. This linkage is stable and attaches the peptide in a defined orientation. Addition of ~20 peptides/KLH is typically obtained by this conjugation method. The longer, full length Aβ peptides were linked to carrier proteins using a glutaraldehyde coupling procedure.

β-amyloid Antigen Cocktail. The three Aβ peptides shown in Figs. 2-4 were each individually conjugated to KLH. 20 μg of each of these three conjugates was then mixed together. This mixture was emulsified with complete Freunds adjuvant and injected i.p. into mice. Subsequent monthly i.p. booster injections used the same cocktail mixture emulsified in incomplete Freunds adjuvant. Control mice received a similar immunization protocol but using KLH which had not been conjugated with the Aβ peptides.

Immunization of Mice. Normal BALB/c mice were immunized by standard procedures with the KLH-linked Aβ vaccines described above. Briefly, mice were injected i.p. with antigen emulsified in complete Freunds adjuvant, followed by a second course in incomplete Freunds adjuvant. The mice were i.v. boosted with antigen in PBS three days prior to bleeding them or removing the spleen for hybridoma fusions to produce monoclonal antibodies.

None of the mice immunized with Aβ vaccines or the anti-Aβ ascites-producing mice displayed ill effects even though some of the antibodies cross-reacted with mouse Aβ and mouse amyloid precursor protein.

ELISA. The presence of bound anti-peptide antibodies was revealed by using a peroxidase-labeled anti-mouse IgG probe followed by the chromogenic substrate (Engvall et al., *Immunochemistry* 8: 871-875 (1971)).

Binding Assay. Both $A\beta_{1-43}$ and $A\beta_{1-40}$ were radiolabeled with ^{125}I. The iodinated peptide was separated from unlabeled material by HPLC to give essentially quantitative specific activity (~2000 Ci/mmol) (Maggio et al., *Proc. Natl. Acad. Sci.* 89: 5462 (1992)). ^{125}I-$A\beta_{1-43}$ probe was incubated for 1h at 23°C with Hy media taken from hybridoma clones producing monoclonal anti-Aβ antibodies. A standard polyethylene glycol separation method was used to detect the amount of ^{125}I-$A\beta_{1-43}$ bound to antibody.

β-Amyloid Vaccines for Primates. The immunogen used was a sythetic Aβ peptide encompassing amino acids 1-41 of the Aβ protein. This peptide was purified by HPLC and freeze-dryed and then resuspended in sterile water at a concentration of 1.5 mg/ml. The vaccine was prepared by mixing 7.5 ml of a 2% aluminum hydroxide gel adjuvant (Alhydrogel, Superfos Biosector, Denmark), referred to herein as alum gel, with 7.5 ml of the peptide. Tests showed that all of the peptide was adsorbed to the alum gel after mixing for 12 hours at 25°C.

Monkeys were initially vaccinated by intramuscular (i.m.) injection of 0.5 ml of the alum-adsorbed peptide. A second vaccination (boost) of the same vaccine preparation (0.5 ml) was administered a month later. Subsequent identical monthly injections (boosts) will be given until the experiment is terminated.

Genetically Engineered Vaccines. Highly immunogenic carrier moieties will be used to construct chimeric Aβ vaccines. Moieties used will include diphtheria toxoid (DT) and the hepatitis B core antigen (HBcAg). The HBcAg expression system will be utilized (Schodel et al., *Infect. and Immun.* 57: 1347-1350 (1989); Schodel et al., *J. of Exper. Med.* 180: 1037-1046 (1994); Schodel et al., *J. of Virology* 66: 106-114 (1992); Milich et al., *Annals New York Academy of Sciences:* 187-201 (1993)). The amino terminal end of the catalytic domain of HBcAg has a

signal sequence which should allow the Aβ fusion protein to be secreted into the culture medium. The culture medium will be concentrated using a large Amicon ultrafiltration device, and the concentrate then chromatographed on a large Superdex 75 column. Recombinant products obtained from within lysed cells will be separated from bacterial protein using a combination of anion exchange and size exclusion FPLC.

SECTION II: ELICITING MONOCLONAL ANTIBODIES WITH TRANSITION STATE ANTIGENS

Transition state peptide antigens

Different types of transition state peptide antigens were synthesized to use in the generation of antibodies which preferentially recognize (hydrolysis) transition states of Aβ at a predetermined amide linkage position.

A series of statine (Sta) transition state analogs encompassing the carboxy-terminal region of Aβ (Cys-Met-Val-Gly-Gly-Val/Sta-Val/Sta-Ile/Sta-Ala-Thr) were synthesized. Replacement of the proposed scissile peptide linkage between Val_{39} and Val_{40}, Val_{40} and Ile_{41}, and Ile_{41} and Ala_{42}, with a "statyl" moiety (-CHOH-CH_2-CO-NH-) was designed to elicit catalytic antibodies that hydrolytically cleave Aβ at one of these sites (Fig. 6). A Cys residue was placed at the N-terminal position of these peptides to provide a suitable linkage group for coupling to a maleimide-activated carrier protein.

A series of phenylalanine statine (PhSta) transition state analogs encompassing the central region of Aβ (Cys-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe/PhSta-Phe/PhSta-Ala-Glu-Asp-Val-Gly-amide) was synthesized in this laboratory.

Replacement of the proposed scissile peptide linkage between Phe_{19} and Phe_{20}, and between Phe_{20} and Ala_{21}, with a statyl moiety (-CHOH-CH_2-CO-NH-) was designed to elicit catalytic antibodies that hydrolytically cleave Aβ at

these sites (Fig. 7). A Cys residue was placed at the C-terminus of these peptides to provide a sulfhydryl linkage group for coupling the peptides to antigenic, maleimide-activated carrier proteins such as KLH.

A structural comparison (Fig. 8) was made between the native Aβ peptide and the transition state phenylalanine statine Aβ peptide using a graphics workstation. An energy minimization algorithm (2000 iterations) was applied to arrange each peptide in its most favorable conformation.

The peptide link (-CO-NH-) between Phe_{19} and Phe_{20} was replaced with an elongated "statyl" moiety (-CHOH-CH_2-CO-NH-) and an energy minimization was applied. This orientation shows the difference between the planar peptide link (-CO-NH-) of natural Aβ (left) versus the extended, tetrahedral "statyl" moiety (-CHOH-CH_2-CO-NH-) in the transition state peptide (right).

An antibody combining site complementary to a tetrahedral statine transition state analog will force the planar peptide bond of the Aβ substrate into a transition state-like conformation. Such distortion should catalyze the cleavage of Aβ at that locus in the peptide sequence.

The possibility of using a reduced peptide bond linkage to mimic the transition state during hydrolysis of an amide linkage was also explored. A reduced peptide bond linkage can be easily placed at almost any site in the Aβ molecule to produce a reduced peptide bond transition state analog. This analog can also be used to elicit catalytic antibodies that will hydrolytically cleave Aβ at the chosen site. The reduced peptide bond transition state Aβ analog made was the (Gln-Lys-Leu-Val-Phe-CH_2-NH_2^+-Phe-Ala-Glu-Asp-Val-Gly-Cys-amide) central region peptide; [calculated 1,342 (M+1); observed 1,344].

A structural comparison (Fig. 9) was made between the native Aβ peptide and the reduced peptide bond transition state Aβ analog using a graphics workstation.

The peptide link (-CO-NH-) between Phe_{19} and Phe_{20} was replaced with a reduced peptide bond ($-CH_2-NH_2^+-$) and an energy minimization was applied. The orientation shown indicates the difference between the planar peptide link (-CO-NH-) of natural Aβ (left) versus the corresponding tetrahedral moiety ($-CH_2-NH_2^+-$) in the reduced peptide bond transition state analog (right). An energy minimization algorithm (2000 iterations) was applied to arrange each peptide in its most favorable conformation.

A phosphonamidate transition state analog of the carboxy-terminal region of Aβ has also been synthesized (Fig. 10). Replacement of the proposed scissile peptide linkage between Gly_{38} and Val_{39} with a phosphonamidate moiety ($-PO_2^-$-NH-) was designed to elicit catalytic antibodies that will hydrolytically cleave Aβ at this site. The N-acetyl-Cys residue was placed at the position of Leu_{34} to provide a suitable linkage group for coupling this peptide to an antigenic carrier protein. The structures in Figure 11 represent the putative transition state for peptide hydrolysis by zinc peptidases, versus structure of and the phosphonate and phosphonamidate mimics. Similar tetrahedral transition state intermediates are known to be formed by reaction with each of the four classes of proteolytic enzymes, the serine-, cysteine-, aspartic- and metallo-peptidases.

A structural comparison was made between the native Aβ peptide and the transition state phosphonamidate Aβ peptide (Fig. 12) using a graphics workstation. The peptide link (-CO-NH-) between Gly_{38} and Val_{39} was replaced with a phosphonamidate bond ($-PO_2^-$-NH-) and an energy minimization was applied. The orientation shown in Figure 12 illustrates the difference between the planar peptide link (-CO-NH-) of native Aβ (left) versus the corresponding tetrahedral phosphonamidate bond ($-PO_2^-$-NH-) in the transition state peptide (right).

An antibody combining site complementary to the tetrahedral transition state analog on the right of Fig.

12, will force the normally planar bond of the Aβ substrate peptide on the left into a transition state-like conformation. Such bond distortion is expected to catalyze the hydrolytic cleavage of the Aβ peptide at the Gly_{38}-Val_{39} linkage.

Immunization with Transition State Peptide Antigens

Peptide antigens were coupled to the immunogenic carrier KLH prior to immunization of mice. Standard protocols were used to immunize BALB/c mice with the KLH-linked Aβ peptides described in the preceding sections. Briefly this procedure used i.p. injection of the different antigens emulsified in complete Freunds adjuvant, followed by a second course in incomplete Freunds adjuvant. Three days prior to hybridoma fusion, the BALB/c mice were boosted i.v. with antigen in PBS.

A hybridoma fusion was performed using the spleen of a mouse immunized with either a mixture of the phenylalanine statine transition state antigens generated (Fig. 7), a mixture of the statine (Sta) transition state Aβ antigens generated (Fig. 6), the reduced peptide bond transition state Aβ antigen generated (transition state mimic located between Phe_{19}-Phe_{20}), or the phosphoamidate transition state Aβ antigen generated (transition state mimic located between Gly_{38}-Val_{39}). Monoclonal antibodies listed in Table 8 were generated from these mice.

Table 8.

Analog Used	Bonds Modified	Potential Cleavage Sites	Antibodies Generated
statine	Val_{39}-Val_{40} Val_{40}-Ile_{41} Ile_{41}-Ala_{42}	Val_{39}-Val_{40} Val_{40}-Ile_{41} Ile_{41}-Ala_{42}	2B2, 2H6, 3F2, 4D3, 6A6, 1E4, 11E9, 9D6, 5C7, 7C7, 1D12
phenylalanine-statine	Phe_{19}-Phe_{20} Phe_{20}-Ala_{21}	Phe_{19}-Phe_{20} Phe_{20}-Ala_{21}	6E2, 5A11, 6F11, 2E3, 8E3, 5G4, 4C7, 8D12, 2C12, 4G7, 5C7, 3C1, 4H9, 8E6, 1H2, 3B1, 2H11
reduced peptide	Phe_{19}-Phe_{20}	Phe_{19}-Phe_{20}	6E7, 6F6

bond			
phospho-amidate	Glu_{38}-Val_{39}	Gly_{38}-Val_{39}	in progress

Demonstration of Aβ Binding by Generated Antibodies

It was very important to demonstrate that the anti-Aβ and anti-transition state Aβ monoclonal antibodies bound to the natural $A\beta_{1-43}$ peptide which they were designed to sequester or cleave. To do this, $A\beta_{1-40}$ and $A\beta_{1-43}$ were radiolabeled with ^{125}I and the iodinated peptide was then separated from unlabeled material by HPLC. Probe was incubated with either purified anti-Aβ antibodies or media taken from hybridoma clones producing anti-Aβ antibodies. The amount of ^{125}I-$A\beta_{1-43}$ bound to antibody was determined using a polyethylene glycol separation method. Results of the experiment are presented in Table 3.

The data in Table 3 demonstrate the ability of the purified 5A11 monoclonal anti-Aβ antibody to bind a high percent of ^{125}I-$A\beta_{1-40}$. This binding assay was used to screen clones and purified antibodies (Table 3) for their ability to bind Aβ. Similar procedures can also serve as the basis for a competitive displacement assay to measure the relative binding strength of different unlabeled Aβ peptides. (Note: with very efficient catalytic antibodies this binding assay may have to be performed on ice to ensure that no cleavage of Aβ occurs during the 1h incubation time.) The assay rapidly identified clones producing high affinity anti-Aβ antibodies.

Monoclonal antibodies from hybridomas obtained using the phenylalanine statine transition state Aβ-KLH antigen were screened by ELISA to assess their binding to both the normal $A\beta_{1-43}$ peptide and to the phenylalanine statine transition state Aβ peptide. Two major patterns were found (Fig. 13).

One group of antibodies (the left portion of Fig. 13) bound to the immunizing transition state peptide and cross-reacted strongly with the native $A\beta_{1-43}$ peptide (each was adsorbed directly onto the ELISA plate). The second group (the right portion) showed a high binding preference for the phenylalanine statine transition state $A\beta$ peptide and reacted minimally with native $A\beta_{1-43}$.

Strong color reactions were obtained in this ELISA using only 10 μl of hybridoma supernatant while Hy media alone or PBS gave a low background (Fig. 13). These results demonstrate that the comparative ELISA screen, although only a semi-quantitative measure of binding, provides a means for identifying monoclonal antibodies that are highly selective for, and most reactive with, the transition state. Importantly, the experiment was performed with carrier-free $A\beta$ peptides adsorbed directly onto microtitre plates, indicating antibody specificity for $A\beta$ peptide rather than carrier.

These findings indicate that several of the generated anti-$A\beta$ transition state antibodies were unique. They bound to both the phenylalanine statine- and normal-$A\beta$ peptides. Their selective recognition of the transition state and weaker cross-reaction with native $A\beta_{1-43}$ however indicates that this binding interaction is very different from that shown by conventional anti-native $A\beta$ antibodies. It further indicates that these new antibodies may be able to force the native $A\beta$ peptide into a conformation resembling the transition state for hydrolytic cleavage. Importantly, some of the antibodies which showed only minimal binding to $A\beta_{1-43}$ in this ELISA, did display cross-reactivity with the natural peptide using a highly sensitive ^{125}I-$A\beta_{1-43}$ binding assay (Table 3).

ELISAs were also performed to investigate the binding of anti-statine analog antibodies to both the normal $A\beta_{1-43}$ peptide and to the statine transition state

Aβ peptide (Fig. 14). The antibodies bound to the C-terminal locus on these carrier-free Aβ peptides (adsorbed directly to the microtitre plate) confirming their anti-peptide specificity. Most of the antibodies preferentially recognized the statine Aβ transition state, but cross-reacted with native $A\beta_{1-43}$. This indicates that these new antibodies are able to force the native Aβ peptide into a conformation resembling the transition state for hydrolytic cleavage of its C-terminal amino acids. Such cleavage is predicted to convert $A\beta_{1-43}$ into potentially less harmful shorter peptides, like $A\beta_{1-40}$ or $A\beta_{1-39}$.

Clone 11E9 had the strongest preference for the statine analog and may be the most likely to have catalytic activity (Fig. 14). Several clones displayed no difference in their reactivity with the native versus statine transition state Aβ peptide. The clones were also tested with $A\beta_{1-40}$ to identify antibodies which do not react with this shortened, 40 amino acid version of Aβ (Fig. 14). Used therapeutically, such antibodies should preferentially bind/cleave the less abundant, but more noxious $A\beta_{1-43}$ species in the blood as opposed to the smaller and less detrimental $A\beta_{1-40}$.

Solid Phase and TLC Aβ Proteolytic Assays

A solid phase ^{125}I-labeled Aβ assay was developed to screen anti-transition state antibody hybridoma supernatants for specific proteolytic activity. The peptide Cys-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Tyr-amide (SEQ ID NO: 5) which encompasses amino acids 14-25 of Aβ was radiolabeled and coupled to a thiol-reactive, iodoacetyl-Sepharose gel to form an irreversible linkage. The product was incubated with anti-transition state antibody and assayed for the progressive release of soluble ^{125}I-peptide from the solid phase matrix. Release of radioactivity from the ^{125}I-Aβ-

Sepharose was used to identify catalytic activity (Fig. 15). The assay was verified by the ability of several different proteases to rapidly hydrolyze this Sepharose-linked Aβ substrate. The peptide was readily accessible to proteolytic cleavage as revealed by a release of soluble ^{125}I-peptide that increased with incubation time.

The results presented in Figure 15 indicate that the antibody-containing media of several clones released ^{125}I-peptide at a greater rate than other clones from this fusion or the PBS and Hy medium controls. Large amounts of these antibodies can be obtained, purified and tested at higher concentrations to achieve much faster rates of cleavage and to verify that the antibodies are acting in a catalytic mode using conventional enzyme kinetics. By changing the composition of the ^{125}I-peptide this same strategy can be used to assay antibodies reactive with different regions of Aβ.

A thin layer chromatography-based autoradiography assay was devised to obtain more definitive evidence for antibody-mediated cleavage of Aβ. Selected anti-phenylalanine statine Aβ transition state clones were expanded and ascites production induced. The different monoclonal antibodies were isolated using protein A-Sepharose. Two ^{125}I-labeled peptides, $A\beta_{1-40}$ and a 17-mer, encompassing amino acids 9-25 of Aβ, were used to test for peptide cleavage. The antibodies were added to the ^{125}I-peptides, allowed to incubate and the reaction mix spotted onto polyamide thin layer sheets which were then developed in different solvents. The migration of ^{125}I-products was followed by exposing the sheet using a quantitative phosphoimager system. Quantitation of the different labeled peptide fragments produced indicated that addition of the antibodies to the Aβ peptides lead to significant break down of the Aβ peptides compared to the untreated peptides (PBS).

Disaggregation of β-amyloid by Monoclonal Antibodies

The self-aggregation of synthetic Aβ peptides has been shown previously to lead to microscopic structures resembling amyloid plaques in the brain (Solomon et al., *Proc. Natl. Acad. Sci. USA* 94: 4109-12 (1997); Solomon et al., *Proc. Natl. Acad. Sci. USA* 93: 452-5 (1996)) which exhibit the same bright green fluorescence upon exposure to thioflavin T. These aggregates are very stable and usually require harsh detergents or strong acids to dissolve. However, it has been demonstrated that the binding of certain anti-Aβ monoclonal antibodies can effectively inhibit the initial aggregation of this peptide and also disaggregate preformed Aβ complexes (Solomon et al., *Proc. Natl. Acad. Sci. USA* 94: 4109-12 (1997); Solomon et al., *Proc. Natl. Acad. Sci. USA* 93: 452-5 (1996)).

A radioactive assay was used to quickly screen the different monoclonal antibodies produced by the present experiments for an ability to dissolve preformed Aβ aggregates, made with ^{125}I-labeled and unlabeled soluble Aβ peptide. An aliquot of the labeled aggregate was incubated with either PBS, the 5A11 anti-Aβ antibody or an equal amount of an irrelevant mouse antibody (7D3, anti-human transferrin receptor), and the level of released radioactivity was subsequently measured (Table 9). The Aβ-specific 5A11 antibody solubilized 80% of the Aβ aggregates while an equal amount of the control antibody had only a minor effect, suggesting that the equilibrium was displaced by antibody-mediated binding of soluble Aβ.

Table 9 Solubilizati n of ^{125}I-Aβ_{1-40} Aggregate by Monoclonal Anti-Aβ Antibody

Addition	^{125}I-Aβ_{1-40} in Ppt. (cpm)	Amount Solubilized (% of PBS Control)
PBS control	3,420	-
+ 5A11 anti-Aβ	676	80
+ 7D3 anti-TfR	2,458	27

Production of Vectorized Anti-Aβ/Anti-Receptor Bispecific Antibodies

Anti-Aβ antibodies were linked to anti-transferrin receptor antibodies (anti-TfR) which served as vectors for delivery of the anti-Aβ antibodies into the brain. The 7D3 mouse monoclonal antibody was used as the anti-TfR part of the construct. 7D3 is specific for the human receptor and selectively immunostains cortical capillaries in normal human brain tissue (Recht et al., *J. Neurosurg.* 72: 941-945 (1990)). Antibody attachment to the receptor is not blocked by an excess of human transferrin. The epitope recognized by this antibody is therefore distant from the receptor-ligand binding site. Bispecific antibodies constructed with this 7D3 antibody and an anti-Aβ antibody are predicted to be useful for therapy in patients with Alzheimer's disease.

For studies in mouse models of Alzheimer's disease an anti-mouse transferrin receptor monoclonal antibody produced in the rat was obtained. This antibody also appears to recognize a transferrin receptor epitope which does not involve ligand binding. The antibody therefore has no effect on cell proliferation when using murine lines.

A series of functional assays were performed after completion of the synthesis, purification and size analysis of the anti-Aβ/anti-transferrin receptor

bispecific antibodies. The vectorized bispecific antibody, composed of a rat monoclonal antibody directed against the mouse transferrin receptor plus the 5A11 mouse anti-Aβ monoclonal antibody, was tested for the ability to attach to transferrin receptor bearing mouse cells. Both components of the bispecific antibody were detected on the cell membrane by cytofluorimetry (Fig. 16) when this duplex was reacted with transferrin receptor positive mouse cells and probed using either a rat IgG-specific or mouse IgG-specific fluorescent secondary antibody reagent.

The capacity of the hybrid reagent to bind ^{125}I-Aβ compared favorably with that of the parent anti-Aβ antibody (Table 10).

Table 10 **^{125}I-Aβ Binding to Bispecific Antibody**

Addition	^{125}I-Aβ_{1-40} Bound (cpm)
Control	4,199
+ anti-Aβ	23,301
+ anti-Aβ/anti-receptor	22,850

To ensure that both of these binding activities resided on the bispecific antibody, transferrin receptor positive cells were treated with the hybrid reagent, unbound material was washed away, and then the cells with bound antibody was exposed to ^{125}I-Aβ_{1-40}. After washing away unbound Aβ, the cell-bound radioactivity was compared to control cells which had been identically prepared except for omission of pretreatment with bispecific antibody. The results are presented in Table 11, and verify the dual specificity of this bispecific antibody by clearly showing that it can simultaneously attach to the cell membrane and bind ^{125}I-Aβ_{1-40}.

Table 11 Bispecific Antibody-Mediated Binding of ^{125}I-Aβ to Receptor-Positive Cells

Pretreatment of Cells	^{125}I-Aβ_{1-40} Bound (cpm)
None	2,367
+ anti-Aβ/anti-transferrin receptor	11,476

Transcytosis of Bispecific Antibody into the Brain

A rat monoclonal anti-mouse transferrin receptor antibody was coupled to a mouse monoclonal antibody (obtained from American Type Culture Collection (ATCC TIB 219), also designated R17 217.1.3 (*Cell. Immunol.* 83: 14-25 (1984)), so that the entry of this new vectorized bispecific construct into brain could be monitored. The bispecific antibody was labeled with ^{125}I and injected i.v. into normal mice. After different lengths of time the mice were sacrificed and the amount of ^{125}I-bispecific antibody that crossed the blood-brain barrier and entered the brain was gauged by a mouse capillary depletion method (Friden et al., *J. Pharm. Exper. Ther.* 278: 1491-1498 (1996); Triguero et al., *J. Neurochem.* 54: 1882-1888 (1990)).

The amount of vectorized bispecific antibody found in the brain parenchyma or brain capillary fractions was measured following differential density centrifugation of the brain homogenate. These values were plotted as a function of time after i.v. injection (Fig. 17). The time-dependent redistribution of radiolabeled bispecific antibody from the capillaries and into the parenchyma was consistent with its passage across the cerebral endothelial blood-brain barrier (Joachim et al., *Nature* 341: 6239:226-30 (1989)). Even greater accumulation in the parenchyma is expected to occur if the antibodies attach to Aβ in the cerebral plaques of plaque-bearing mice.

Monitoring the Brain Distribution of Bispecific Antibody in Live Mice

The ability to follow the entry and accumulation of vectorized bispecific antibodies in the brain of live mice would greatly assist in the development of the intracerebral treatment of plaque-bearing mice. Such a development would enable time-course studies and would greatly reduce problems with inter-mouse variability. Preliminary studies with ^{125}I-labeled bispecific antibodies were performed to determine if immunoscintigraphy was feasible in this system. As a first step, either the radiolabeled vectorized bispecific antibody (^{125}I-R17/5A11) or a non-vectorized control bispecific antibody were administered to separate mice. Sequential brain images were accumulated at 1, 6, 24 and 48 hours following i.v. administration of the ^{125}I-labeled bispecific antibody probes. Although this technique suffered from a difficulty in determining how much of the signal was due to the levels of blood-borne radioactivity circulating through the brain, significant distinctions were noted in the brain of mice treated with the mouse transferrin receptor reactive bispecific antibody versus those receiving the control bispecific antibody. When the vectorized agent was used, brain levels increased between 1 and 6 hrs and then declined to a much lower level at 24 and 48 hrs. Mice treated with the control displayed no increase between 1 and 6 hrs. The reason for decreased brain levels at 24 hrs and beyond is not known but might be due to dehalogenation of the bispecific antibody probes so that free ^{125}I is released. Alternative methods utilizing radioactive labels such as ^{111}In (Sheldon et al., *Nucl. Med. Biol.* 18: 519-526 (1991)) or ^{99m}Tc (Texic et al., *Nucl. Med. Biol.* 22: 451-457 (1995)) attached to the vectorized bispecific antibody can be utilized in future experiments if the use of iodine presents a technical problem. This imaging technology will be useful for determining if smaller

vectorized bispecific antibodies (eg. $F(ab')_2$) with different physical properties and an altered biodistribution will penetrate into the brain more effectively.

$F(ab')_2$ Heterodimers for Vector-Mediated Transport into the Brain

The introduction of whole antibodies into the brain might be detrimental if they were to fix complement and promote complement-mediated lysis of neuronal cells. The development of smaller vectorized $F(ab')_2$ bispecific reagents is expected to avoid this problem. It has been shown that aggregated Aβ itself can fix complement in the absence of any antibody and that the resulting inflammation may contribute to the pathology of Alzheimer's disease. The possibility of intracerebral antibody having a similar effect would be greatly reduced by eliminating the Fc region of the antibody. Moreover, since coupling of Fab' halves uses the intrinsic hinge region cysteines, no extraneous substituent linkage groups need be added.

Faster or more efficient entry into the brain represents another potential advantage that smaller $F(ab')_2$ or Fv_2 reagents provide for intracerebral delivery. Such modified bispecific agents can be prepared and compared to full-sized hybrid antibodies for their relative effectiveness in reaching the brain, crossing the blood-brain barrier, and affecting Aβ plaque development, by the methods described herein. It is important to note, however, that only minor differences were found when the capacity of differently-sized anti-transferrin receptor bispecific reagents for delivering toxins into cells by receptor-mediated endocytosis was compared (Raso et al., *J. Biol. Chem.* 272: 27623-27628 (1997)). This observation might indicate that little variation will be seen for transcytosis across the brain capillary endothelial cells which form the blood-brain

barrier. At the very least however one would expect the two types of vectorized molecules to have different biodistribution and plasma half-life characteristics (Spiegelberg et al., *J. Exp. Med.* 121: 323 (1965)).

Methods of the Invention

Antigen Synthesis. The statine and phenylalanine statine transition state peptides were synthesized using automated Fmoc chemistry. Fmoc-statine (Sta), [N-Fmoc-(3S,4S)-4-amino-3-hydroxy-6-methyl heptanoic acid] and Fmoc-"phenylalanine statine" (PhSta), [N-Fmoc-(3S,4S)-4-amino-3-hydroxy-5-phenylpentanoic acid] were purchased commercially. Each peptide was tested for purity by HPLC and its composition was verified by mass spectral and amino acid analysis.

The design strategy and methods for synthesizing phosphonamidate- and phosphonate-based transition state peptides are straightforward (Bartlett et al., *Am. Chem. Society* 22: 4618-4624 (1983); Bartlett et al., *Biochemistry* 26: 8553-8561 (1987)). The N-terminal portion of the peptide (N-acetyl-Cys-Met-Val-Gly) was made using standard automated Fmoc chemistry. After cleavage from the resin the N-acetyl tetrapeptide was treated with pyridine disulfide to protect its sulfhydryl group. An acid chloride of Cbz-glycine phosphonate monomethyl ester (Bartlett et al., *Am. Chem. Society* 22: 4618-4624 (1983); Bartlett et al., *Biochemistry* 26: 8553-8561 (1987)) was coupled with Val-Val-Ile-Ala-amide which was synthesized by automated Fmoc chemistry. The last amino acid of Aβ, Thr, was omitted due to potential problems with its unprotected hydroxyl group. The product, Cbz-Gly-PO_2^--NH-Val-Val-Ile-Ala-amide has a phosphonamidate (methyl ester) bond between the Gly and Val residues. Next, the Cbz blocking group was removed using hydrogen so that the protected N-acetyl-Cys-Met-Val-Gly peptide could be added to the amino terminal end

of this transition state peptide by HBTU-activated peptide linkage. Treatment with mercaptoethanol and rabbit liver esterase was used to deblock the peptide. Each key component in the synthetic scheme was tested for purity by HPLC and its composition was verified by mass spectral and amino acid analysis.

A reduced peptide bond linkage was placed at the indicated sites in the Aβ molecule. Automated Fmoc chemistry was used to begin synthesis of the peptide. A pre-synthesized Fmoc amino aldehyde was then added manually and after the imide was reduced, automated synthesis was resumed (Meyer et al., *J. Med. Chem.* 38: 3462-3468 (1995)).

Coupling of Antigen to Carrier. The native and transition state Aβ peptides were coupled to maleimide-activated KLH by standard procedures (Partis et al., *J. Pro. Chem.* 2: 263-277 (1983)), in order to elicit an immune response. A Cys residue was strategically placed at the N- or C-terminal end of the peptides to provide a suitable linkage group for coupling them via a thioether bond to maleimide activated carrier proteins. This stable linkage attaches the peptide in a defined orientation. Addition of ~20 peptides/KLH has been obtained based upon the transition state amino acid content as determined by amino acid analysis of the hydrolyzed conjugates (Tsao et al., *Anal. Biochem.* 197: 137-142 (1991)).

Immunization of Mice. Standard protocols were used to immunize mice with the KLH-linked Aβ peptides described in the preceding sections. Briefly this procedure used i.p. injection of the different antigens emulsified in complete Freunds adjuvant, followed by a second course in incomplete Freunds adjuvant. Three days prior to the hybridoma fusion, the BALB/c mice were boosted i.v. with antigen in PBS.

After ~1 month animals were given a boost i.p. using the antigen emulsified with incomplete adjuvant. Serum from these animals was analyzed for anti-peptide antibodies by ELISA. BALB/c mice showing abundant antibody production were boosted by an i.v. injection with antigen and three days later they were used to generate hybridoma clones that secrete monoclonal antibodies.

None of the mice immunized with Aβ vaccines or the anti-Aβ ascites-producing mice displayed ill effects even though some of those induced antibodies cross-react with mouse Aβ and mouse amyloid precursor protein.

Hybridoma Production I. A hybridoma fusion was performed using the spleen of a mouse immunized with the phenylalanine statine transition state Aβ-KLH antigen. Spleen cells from mice with the highest titre were fused with mouse myeloma NS-1 cells to establish hybridomas according to standard procedures (Köhler et al., *Nature* 256: 495 (1975); R.H. Kennett, Fusion Protocols. Monoclonal Antibodies, eds. R.H. Kennett, T.J. McKearn and K.B. Bechtol. Plenum Press, New York. 365-367 pp. (1980)).

^{125}I-Aβ Binding Assay. $A\beta_{1-40}$ and $A\beta_{1-43}$ were radiolabeled with ^{125}I and the iodinated peptide then separated from unlabeled material by HPLC to give quantitative specific activity (~2000 Ci/mmol) (Maggio et al., *Proc. Natl. Acad. Sci.* 89: 5462-5466 (1992)). This probe was incubated for 1h at 23°C with either purified anti-Aβ antibodies or media taken from hybridoma clones producing anti-Aβ antibodies. A polyethylene glycol separation method was used to detect the amount of ^{125}I-$A\beta_{1-43}$ bound to antibody. By using serial dilution, this assay can provide relative binding affinities for the different hybridoma supernatants or purified antibodies.

Solid Phase Aβ Proteolytic Assay. A solid phase ^{125}I-labeled Aβ assay was developed to screen anti-transition state antibody hybridoma supernatants for specific proteolytic activity. The Cys-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Tyr-amide peptide (SEQ ID NO: 5) encompassing amino acids 14-25 of Aβ was radiolabeled with ^{125}I and the iodinated peptide was then separated from unlabeled material by HPLC. The highly radioactive Aβ peptide was coupled to a thiol-reactive, iodoacetyl-Sepharose gel to form an irreversible linkage. Antibodies were added to the labeled Aβ, which was then assayed for progressive release of soluble ^{125}I-peptide from the solid phase matrix at pH 7, 25°C. This assay was verified by the ability of several different proteases in to rapidly hydrolyze this Sepharose-linked Aβ substrate. Release of soluble ^{125}I-peptide increased with incubation time.

Although Aβ is cleaved by several naturally occurring proteases, preliminary tests indicated that interference from high levels of background hydrolysis was not a problem when assaying hybridoma supernatants of clones that did produce catalytic antibodies. A further precaution that can be taken against exogenous proteases is carrying out all hybridoma cell fusions and cell culturing in serum-free media.

TLC Aβ Proteolytic Assay. A thin layer chromatography-based autoradiography assay was used to obtain more definitive evidence for antibody-mediated cleavage of Aβ. Selected anti-phenylalanine statine Aβ transition state clones were expanded and ascites production induced. The different monoclonal antibodies were isolated using protein A-Sepharose. The cleavage assay used ^{125}I-Aβ_{1-40} and an ^{125}I-labeled 17-mer, encompassing amino acids 9-25 of Aβ. Binding of the two ^{125}I-labeled peptides to the purified monoclonal antibodies 5A11 and 6E2 was examined using either a PEG precipitation assay or by a co-

electrophoresis method. Peptide cleavage was tested by adding the antibodies to the ^{125}I-peptides, incubating and then spotting the reaction mix onto polyamide thin layer sheets. The chromatographs were developed in different solvents (eg. 0.5 N HCl, 0.5 N NaOH or pH 7 phosphate buffer) and the migration of ^{125}I-products was followed by exposing the sheet using a quantitative phosphoimager system.

Screen and Isolate Select Anti-Aβ Antibodies. An ELISA was used to initially screen for anti-Aβ and anti-transition state Aβ peptide monoclonal antibodies. Both the transition state peptide and the corresponding natural Aβ peptide were adsorbed onto separate microtitre plates. The hybridoma supernatants were screened using two assays so that the relative binding to both native and transition state Aβ peptides could be quantitated. Clones producing monoclonal antibodies that preferentially recognized the transition state or bound Aβ with high affinity were selected for expansion and further study.

Propagation and Purification of Monoclonal Antibodies. Selected clones producing anti-Aβ antibodies and clones producing anti-receptor antibodies were injected into separate pristane-primed mice. Ascites were collected and the specific monoclonal antibodies isolated. Purification of antibodies from ascites was accomplished using a Protein A column or alternatively, antibodies were isolated from ascites fluid by $(NH_4)_2SO_4$ precipitation and passage over an S-300 column to obtain the 150 kDa immunoglobulin fraction. Monovalent Fab fragments were prepared and isolated by established methods. Their purity was evaluated by SDS-PAGE under reducing and non-reducing conditions. 50-100 mg of purified monoclonal antibody was routinely obtained from each ascites-bearing mouse.

Further Characterization of Catalytic Activity on Aβ Substrates. To fully define the hydrolytic properties of the isolated anti-transition state antibodies some very important controls can be run. First the ability to completely block catalytic antibody activity with the appropriate transition state peptide can be verified. This non-cleavable "inhibitor" should bind much more tightly to the antibody combining sites and thereby prevent substrate binding or cleavage. Substrate specificity can be further established by showing no cleavage of a sham Aβ peptide having a different amino acid sequence. The products of hydrolysis can also be fully characterized by HPLC, amino acid and mass spectral analysis. Control antibodies that are not directed against the transition state Aβ can be tested and confirmed to produce no catalysis. Finally, catalytic activity can be shown to reside in the purified Fab fragments of the anti-transition state antibody.

Purified Anti-Aβ Antibodies Dissolve Preformed Aβ Aggregates. (Walker et al., *Soc. Neurosci. Abstr.* 21: 257 (1995), Zlokovic, B.V., *Life Sciences* 59: 1483-1497 (1996)). Aβ precipitates were formed and measured *in vitro* (Yankner et al., *Science* 250: 279-282 (1990), Kowall et al., *Proc. Natl. Acad. Sci.* 88: 7247-7251 (1991)). A radioactive assay was used to quickly screen the different monoclonal antibodies produced for an ability to dissolve preformed Aβ aggregates. After adding ^{125}I-Aβ to unlabeled soluble peptide, aggregates were formed by bringing the solution to pH 5 or by stirring it overnight in PBS. An aliquot of the labeled aggregate was incubated for 1 hr with either PBS, the 5A11 anti-Aβ antibody or an equal amount of an irrelevant mouse antibody (7D3, anti-human transferrin receptor). After centrifugation, the level of radioactivity in the precipitate was measured.

Generation of Vectorized Anti-Aβ/Anti-Receptor Bispecific Antibodies. The anti-Aβ antibodies were chemically coupled to anti-human transferrin receptor and anti-mouse transferrin receptor antibodies by different methods (Raso et al., *J. Biol. Chem.* 272: 27623-27628 (1997); Raso et al., Monoclonal antibodies as cell targeted carriers of covalently and non-covalently attached toxins. *In* Receptor mediated targeting of drugs, vol. 82. G. Gregoriadis, G. Post, J. Senior and A. Trouet, editors. NATO Advanced Studies Inst., New York. 119-138 (1984)). A rapid thioether linkage technique was used to form strictly bispecific hybrids using Traut's reagent and the heterobifunctional SMBP reagent. One component was sparingly substituted with thiol groups (SH). These readily reacted to form a thioether linkage upon mixture with the maleimido-substituted (M) second component following the reaction:

$$Ab_A\text{-}SH + Ab_B\text{-}M \rightarrow Ab_A\text{-}S\text{-}Ab_B$$

Gel filtration of the reaction mixture on an S-300 column yielded the purified dimer which was 300 kDa and had two sites for binding Aβ plus two sites for attachment to transferrin receptors on brain capillary endothelial cells. Non-targeted control hybrids were formed by linking a nonspecific MOPC antibody to the anti-Aβ antibody. This hybrid antibody does bind Aβ, but, being non-reactive with transferrin receptors, should not cross the blood-brain barrier.

$F(ab')_2$ fragments of the two different antibody types can similarly be thioether-linked to form Fc-devoid reagents that cannot bind complement which might otherwise cause neurotoxic effects. These smaller bispecific hybrids (100 kDa) can be formed by reducing the intrinsic disulfides which link the heavy chains of $F(ab')_2$ fragments (Raso et al., *J. Immunol.* 125: 2610-2616 (1980)). The thiols generated are stabilized and

Ellman's reagent (E) is used to activate these groups on one of the components (Brennan et al., *Science* 229: 81-83 (1985)). Exclusively bispecific $F(ab')_2$ hybrids can be formed upon mixing the reduced Fab' with an activated Fab' having the alternate specificity according to the reaction:

$$Fab'_A\text{-}SH + Fab'_B\text{-}SS\text{-}E \rightarrow Fab'_A\text{-}SS\text{-}Fab'_B + E\text{-}SH$$

Purification on an S-200 column will isolate hybrids with one site for binding Aβ and one site for interaction with the target epitope on the brain capillary endothelial cells.

A similar approach can be used to make even smaller disulfide-linked single chain Fv heterobispecific dimers, Fv_A-SS-Fv_B (50 kDa), to cross the blood-brain barrier. Soluble Fvs can be constructed to possess a carboxyl-terminal cysteine to facilitate the disulfide exchange shown in the reaction below, and create 50 kDa heterodimers exclusively:

$$Fv_A\text{-}SH + Fv_B\text{-}SS\text{-}E \rightarrow Fv_A\text{-}SS\text{-}Fv_B + E\text{-}SH$$

In side by side comparisons between whole antibody and either Fab' or Fv based bispecific reagents, the latter have proven to be moderately more effective on a molar basis for cell uptake via the transferrin receptor-mediated pathway (Raso et al., *J. Biol. Chem.* 272: 27623-27628 (1997)). Since these smaller constructs are monovalent for the cell-surface epitope, those findings dispel the notion that cross-linking of two surface receptors is necessary for the cellular uptake of immunocomplexes.

Functional Assays for Dual Binding Activity of Bispecific Antibodies. The capacity of the hybrid reagent to bind ^{125}I-Aβ was compared with that of the parent anti-Aβ

antibody in a standard PEG binding assay (see Table 10 for binding assays).

The ability of the appropriate bispecific antibodies to attach to transferrin receptor bearing human or mouse cells was confirmed by cytofluorimetry. The bispecific antibody was reacted with transferrin receptor positive human or mouse cells and probed using either a rat IgG-specific or mouse IgG-specific fluorescent secondary antibody reagent.

Measurement of Aβ Binding Using ^{125}I-Aβ and a Polyethylene Glycol Separation. To ensure bispecificity, hybrid reagents were tested for a capacity to mediate the attachment of ^{125}I-Aβ to receptor-bearing cells. Transferrin receptor positive cells were treated with the hybrid reagent, washed away unbound material and then exposed these cells to ^{125}I-$A\beta_{1-40}$. The cells were washed and the amount of cell-bound radioactivity was compared to control cells which had been identically prepared except that pretreatment with bispecific antibody was omitted.

Capillary Depletion. The bispecific antibody was labeled with ^{125}I and injected i.v. into normal mice. After different lengths of time the mice were sacrificed and the amount of ^{125}I-bispecific antibody that crossed the blood-brain barrier and entered the brain was gauged by a mouse capillary depletion method (Friden et al., *J. Pharm. Exper. Ther.* 278: 1491-1498 (1996); Triguero et al., *J. Neurochem.* 54: 1882-1888 (1990)). The amount of vectorized bispecific antibody found in the brain parenchyma or brain capillary fractions was measured following differential density centrifugation of the brain homogenate. These values were plotted as a function of time after i.v. injection. Progressive passage from capillaries into the parenchyma indicates active transcytosis across the blood-brain barrier.

Immunoscintigraphy. A non-invasive method for monitoring intracerebral delivery process which involves visualizing the entry of a radiolabeled bispecific antibody into the brain of live mice, can also be used. Radiolabeled vectorized bispecific antibody (^{125}I-R17/5A11) or a non-vectorized control bispecific antibody were administered to separate mice. Sequential brain images were accumulated at 1, 6, 24 and 48 hours following i.v. administration of the ^{125}I-labeled bispecific antibody probes. The animals were chemically immobilized during exposure using ketamine/xylazine anesthesia. This imaging technology could be very useful for determining if circulating anti-Aβ antibodies will prevent i.v. administered ^{125}I-Aβ from entering the brain. Digital scintigraphy data was quantified using standards and the integration functions provided in the analysis software.

CLAIMS

1. An antibody which catalyzes hydrolysis of β-amyloid at a predetermined amide linkage.

2. The antibody of Claim 1 which catalyzes hydrolysis of the amide linkage between residues 39 and 40 of β-amyloid.

3. The antibody of Claim 1 which catalyzes hydrolysis of the amide linkage between residues 40 and 41 of β-amyloid.

4. The antibody of Claim 1 which catalyzes hydrolysis of the amide linkage between residues 41 and 42 of β-amyloid.

5. The antibody of Claim 1 which preferentially binds a transition state analog which mimics the transition state adopted by β-amyloid during hydrolysis at a predetermined amide linkage, and also binds to natural β-amyloid with sufficient affinity to detect using an ELISA.

6. The antibody of Claim 1 which preferentially binds a transition state analog which mimics the transition state adopted by β-amyloid during hydrolysis at a predetermined amide linkage, and does not bind natural β-amyloid with sufficient affinity to detect using an ELISA.

7. A vectorized antibody which is characterized by the ability to cross the blood brain barrier and the ability to catalyze the hydrolysis of β-amyloid at a predetermined amide linkage.

8. The vectorized antibody of Claim 7 which is a bispecific antibody.

9. The vectorized antibody of Claim 8 which has a first specificity for the transferrin receptor and a second specificity for a transition state adopted by β-amyloid during hydrolysis.

10. The vectorized antibody of Claim 9 which catalyzes hydrolysis of β-amyloid between residues 39 and 40.

11. A method for sequestering free β-amyloid in the bloodstream of an animal, comprising the steps:
a) providing antibodies specific for β-amyloid; and
b) intravenously administering the antibodies to the animal in an amount sufficient to increase retention of β-amyloid in the circulation.

12. A method for sequestering free β-amyloid in the bloodstream of an animal, comprising the steps:
a) providing an antigen comprised of an epitope which is present on endogenous β-amyloid; and
b) immunizing the animal with the antigen of step a) under conditions appropriate for the generation of antibodies which bind endogenous β-amyloid.

13. A method for reducing levels of β-amyloid in the brain of an animal, comprising the steps:
a) providing antibodies specific for β-amyloid endogenous to the animal; and
b) intravenously administering the antibodies to the animal in an amount sufficient to increase retention of β-amyloid in the circulation of the animal.

14. The method of Claim 13 wherein the antibodies specific for β-amyloid are catalytic antibodies which catalyze hydrolysis of β-amyloid at a predetermined amide linkage.

15. The method of Claim 13 wherein the antibodies are monoclonal.

16. The method of Claim 13 wherein the antibodies are polyclonal.

17. The method of Claim 13 wherein the antibodies specifically recognize epitopes on the C-terminus of β-amyloid$_{1-43}$.

18. A method for reducing levels of β-amyloid in the brain of an animal, comprising the steps:
    a) providing an antigen comprised of an epitope which is present on β-amyloid endogenous to the animal; and
    b) immunizing the animal with the antigen of step a) under conditions appropriate for the generation of antibodies which bind endogenous β-amyloid.

19. The method of Claim 18 wherein the antigen is a transition state analog which mimics the transition state adopted by β-amyloid during hydrolysis at a predetermined amide linkage.

20. The method of Claim 18 wherein the antigen is comprised of Aβ_{10-25}.

21. The method of Claim 19 wherein the antibodies generated have a higher affinity for the transition state analog than for natural β-amyloid.

22. The method of Claim 19 wherein the antibodies generated catalyze hydrolysis of endogenous β-amyloid.

23. A method for preventing the formation of amyloid plaques in the brain of an animal, comprising the steps:
    a) providing an antigen comprised of an epitope which is present on β-amyloid endogenous to the animal; and
    b) immunizing the animal with the antigen of step a) under conditions appropriate for the generation of antibodies which bind endogenous β-amyloid.

24. The method of Claim 23 wherein the antigen is a transition state analog which mimics the transition state adopted by β-amyloid during hydrolysis at a predetermined amide linkage.

25. A method for reducing levels of circulating β-amyloid in an animal, comprising the steps:
    a) providing an antigen comprised of an epitope which is a mimic of a predetermined hydrolysis transition state of a β-amyloid polypeptide endogenous to the animal; and
    b) immunizing the animal with the antigen of step a) under conditions appropriate for the generation of antibodies to the β-amyloid hydrolysis transition state.

26. A method for reducing levels of circulating β-amyloid in an animal, comprising the steps:
    a) providing antibodies which catalyze the hydrolysis of β-amyloid endogenous to the animal; and

b) intravenously administering the antibodies to the animal.

27. A method for preventing the formation of amyloid plaques in the brain of an animal, comprising the steps:
a) providing antibodies which catalyze hydrolysis of β-amyloid produced by the animal at a predetermined amide linkage; and
b) administering the antibodies to the animal in an amount sufficient to cause a significant reduction in β-amyloid levels in the blood of the animal.

28. A method for reducing levels of β-amyloid in the brain of an animal, comprising the steps:
a) providing vectorized bispecific antibodies competent to transcytose across the blood brain barrier, which catalyze hydrolysis of β-amyloid of the animal at a predetermined amide linkage; and
b) intravenously administering the antibodies to the animal.

29. The method of Claim 28 wherein the vectorized bispecific antibodies specifically bind the transferrin receptor.

30. The method of Claim 28 wherein the vectorized bispecific antibodies catalyze hydrolysis of the amide linkage between residues 39 and 40 of β-amyloid.

31. A method for disaggregating amyloid plaques present in the brain of an animal comprising the steps:

a) providing vectorized bispecific antibodies competent to transcytose across the blood brain barrier, which catalyze hydrolysis of β-amyloid produced by the animal at a predetermined amide linkage; and

b) intravenously administering the antibodies to the animal in an amount sufficient to cause significant reduction in β-amyloid levels in the brain of the animal.

32. A method for disaggregating amyloid plaques present in the brain of an animal, comprising the steps:

a) providing antibodies which catalyze hydrolysis of β-amyloid produced by the animal at a predetermined amide linkage; and

b) administering the antibodies to the animal.

33. A method for generating antibodies which catalyze hydrolysis of a protein or polypeptide comprising the steps:

a) providing an antigen, the antigen being comprised of an epitope which has a statine analog which mimics the conformation of a predetermined hydrolysis transition state of the polypeptide;

b) immunizing an animal with the antigen under conditions appropriate for the generation of antibodies to the hydrolysis transition state.

34. The method of Claim 33 wherein the protein is β-amyloid.

35. A method for generating antibodies which catalyze hydrolysis of a protein or polypeptide comprising the steps:

a) providing an antigen, the antigen being comprised of an epitope which has a reduced peptide bond analog which mimics the conformation of a predetermined hydrolysis transition state of the polypeptide;

b) immunizing an animal with the antigen under conditions appropriate for the generation of antibodies to the hydrolysis transition state.

36. The method of Claim 35 wherein the protein is β-amyloid.

H_2N-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIAT-CO_2H (SEQ ID NO:1)
1 10 20 30 40

FIG. 1

H_2N-Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Cys-amide (SEQ ID NO:2)
1 5 10 15

FIG. 2

Cys-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-amide (SEQ ID NO:3)
10 15 20 25

FIG. 3

Cys-Met-Val-Gly-Gly-Val-Val-Ile-Ala-Thr-CO_2H (SEQ ID NO:4)
35 40

FIG. 4

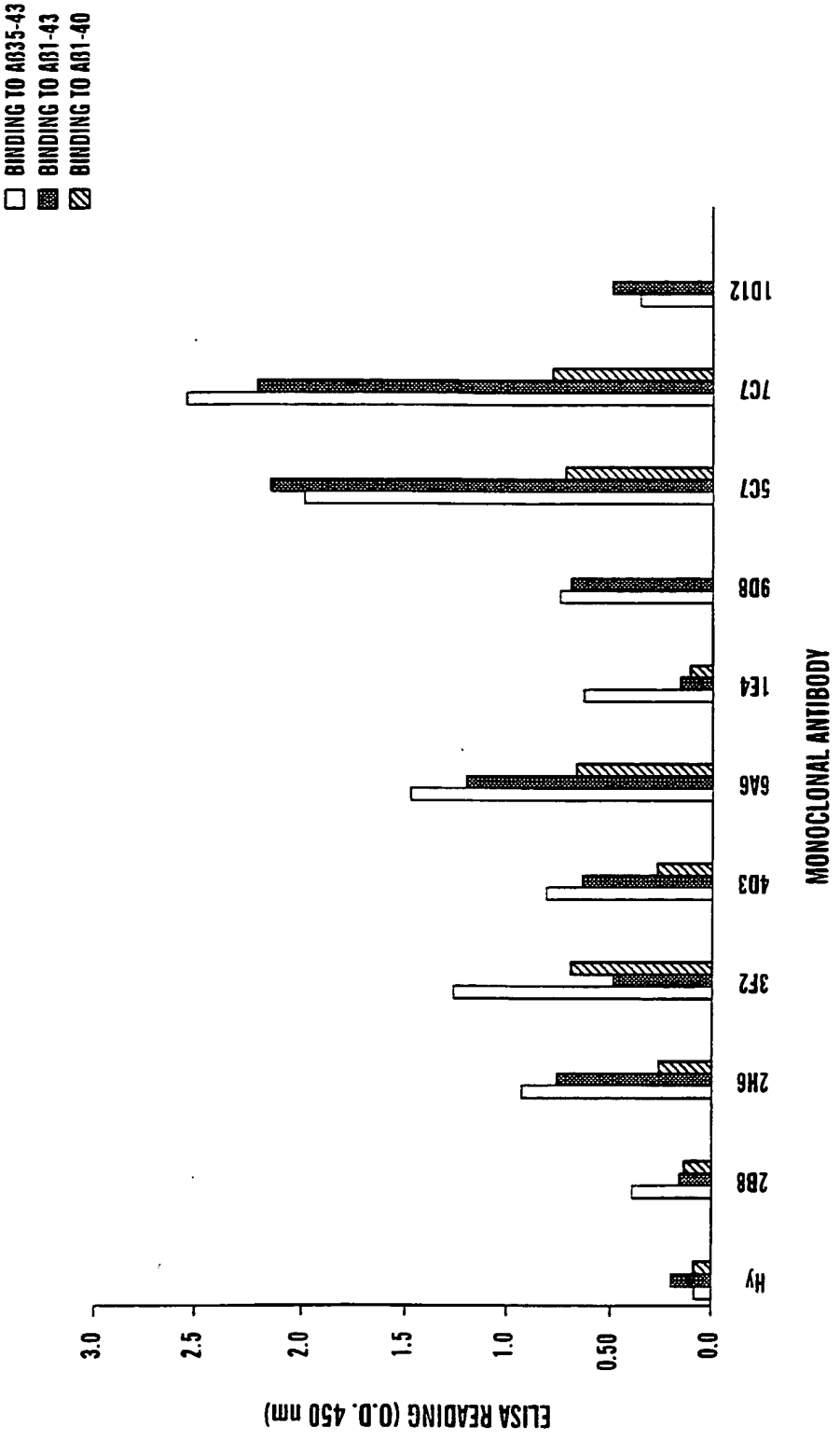

BINDING TO Aβ35-43
BINDING TO Aβ1-43
BINDING TO Aβ1-40
ELISA READING (O.D. 450 nm)
3.0
2.5
2.0
1.5
1.0
0.50
0.0
Hy
2B8
2H6
3F2
4D3
6A6
1E4
9D8
5C7
7C7
10I2
MONOCLONAL ANTIBODY

FIG. 5

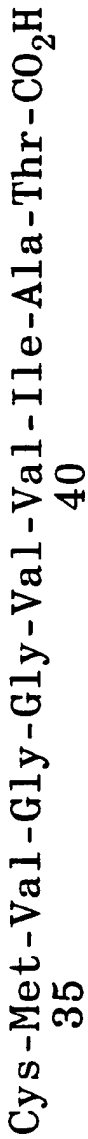

Cys-Met-Val-Gly-Gly-Val-Val-Ile-Ala-Thr-CO_2H
35
40

FIG. 6

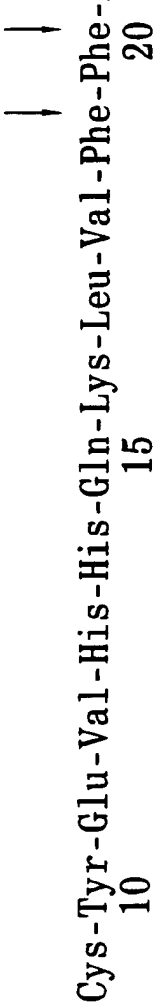
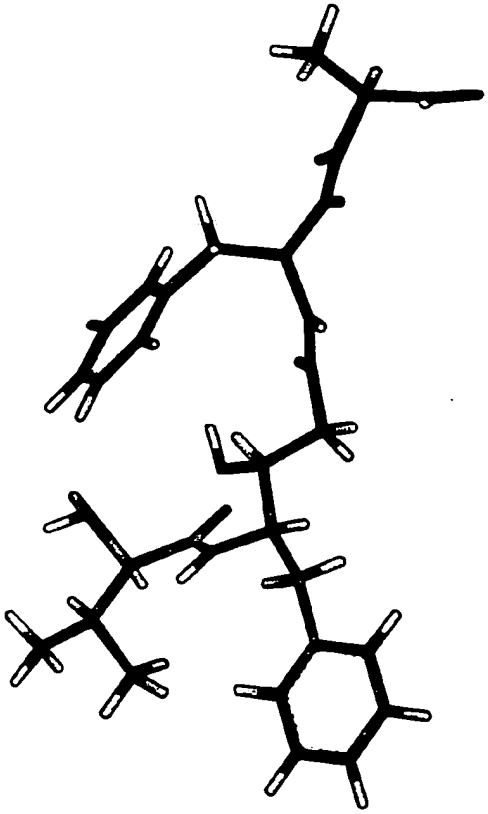
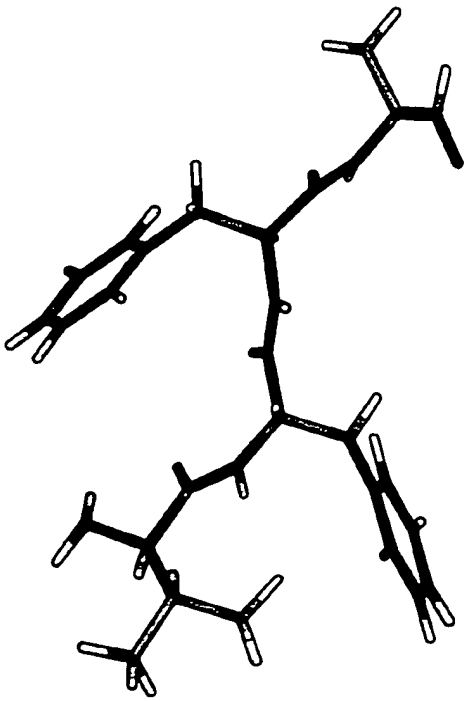

Cys-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-amide
10
15
20
25

FIG. 7



HO—Val-Phe-CH-CH$_2$-CO-NH-Phe-Ala



—Val-Phe-CO-NH-Phe-Ala

FIG. 8

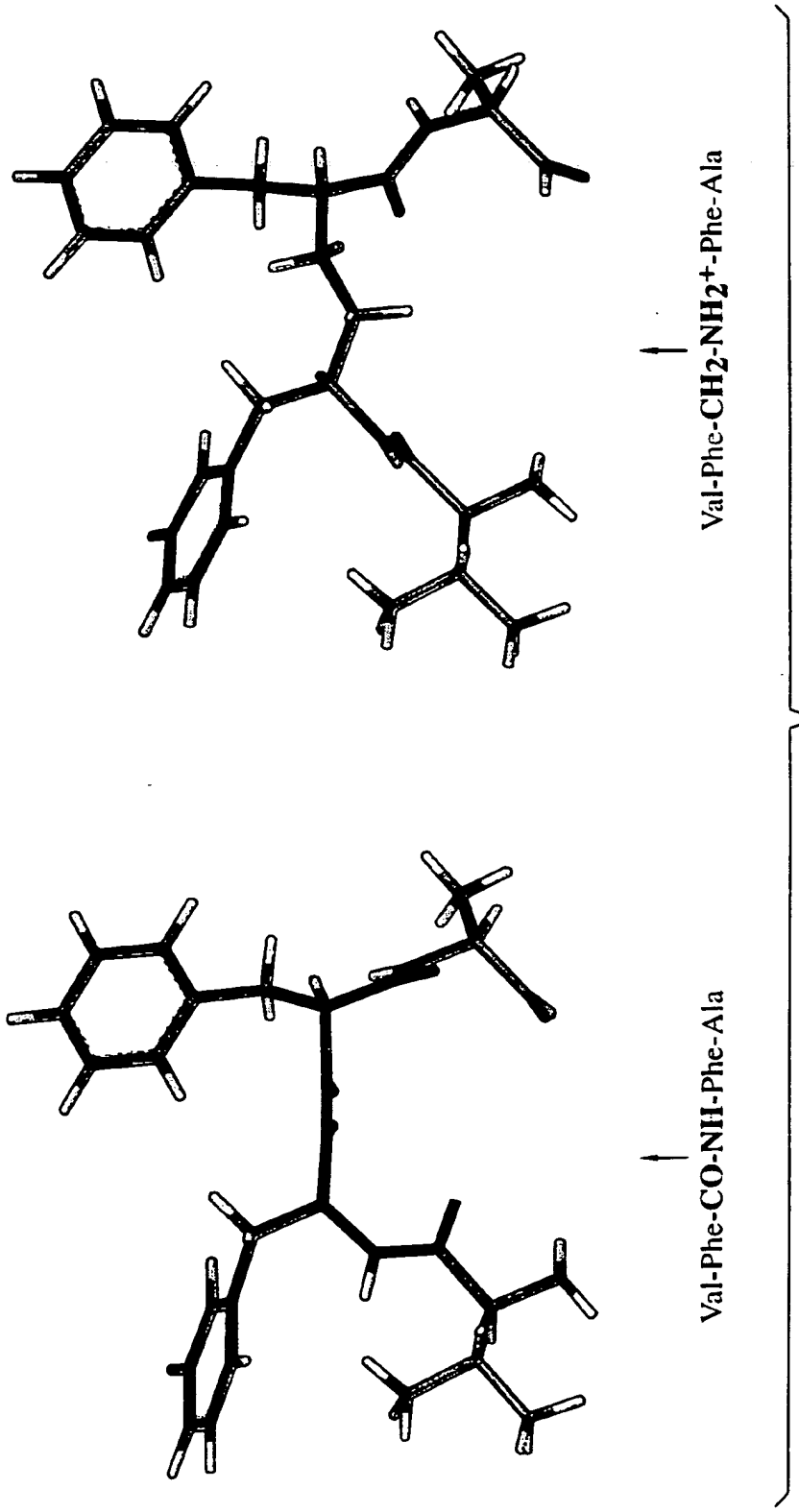
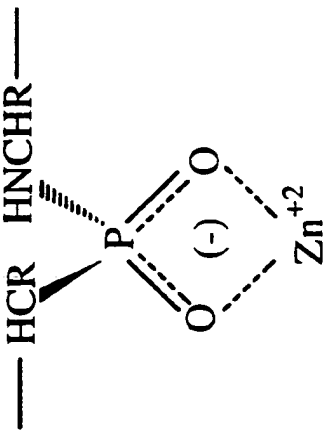


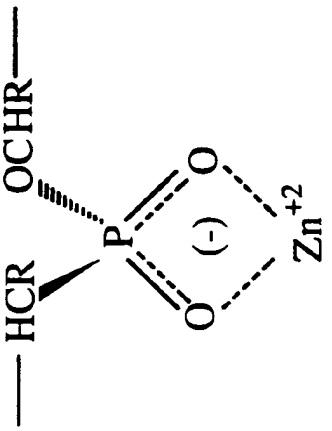
FIG. 9


N-acetyl-Cys-Met-Val-Gly-Gly-CO-NH-Val-Val-Ile-Ala-amide
35
40
N-acetyl-Cys-Met-Val-Gly-Gly-PO2--NH-Val-Val-Ile-Ala-amide

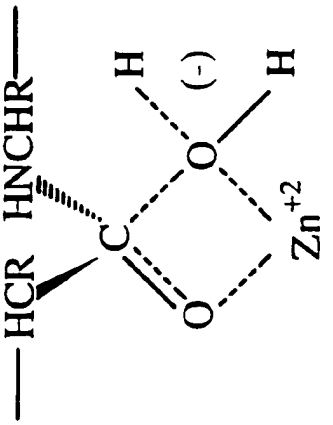
FIG. 10



Phosphonamidate

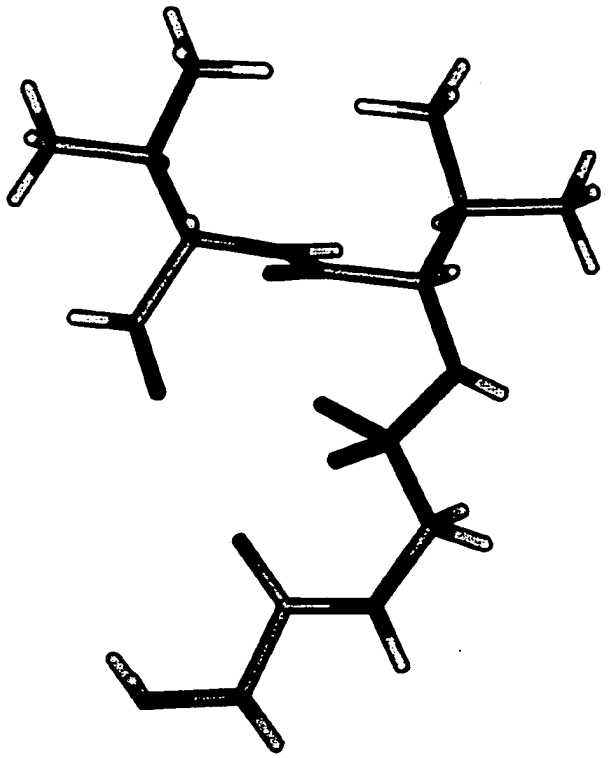


Phosphonate

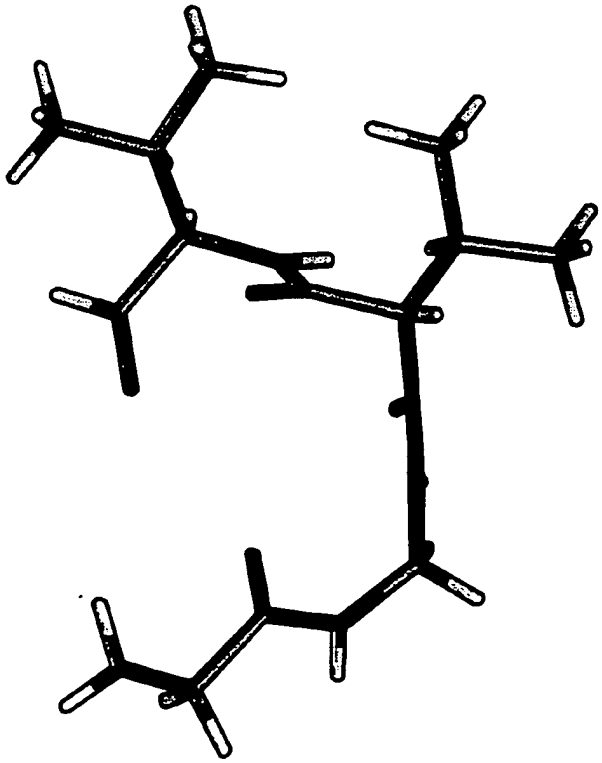


Transition-state

FIG. 11



Gly-Gly-PO_2^--NH-Val-Val



Gly-Gly-CO-NH-Val-Val

FIG. 12

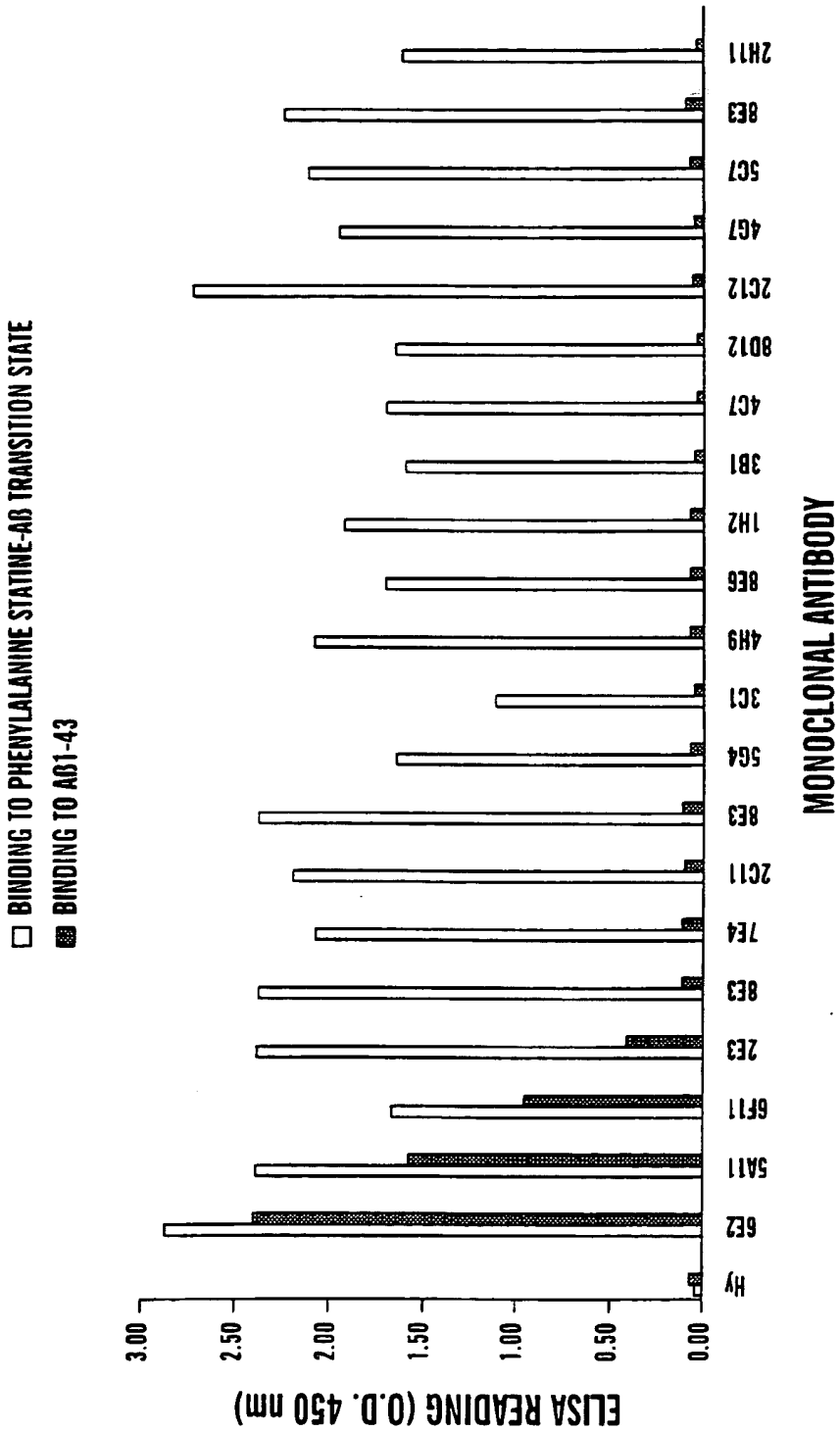

BINDING TO PHENYLALANINE STATINE-AB TRANSITION STATE
BINDING TO Aβ1-43
ELISA READING (O.D. 450 nm)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
HY
6E2
5A11
6F11
2E3
8E3
7E4
2C11
8E3
5G4
3C1
4H9
8E6
1H2
3B1
4C7
8D12
2C12
4G7
5C7
8E3
2H11
MONOCLONAL ANTIBODY

FIG. 13

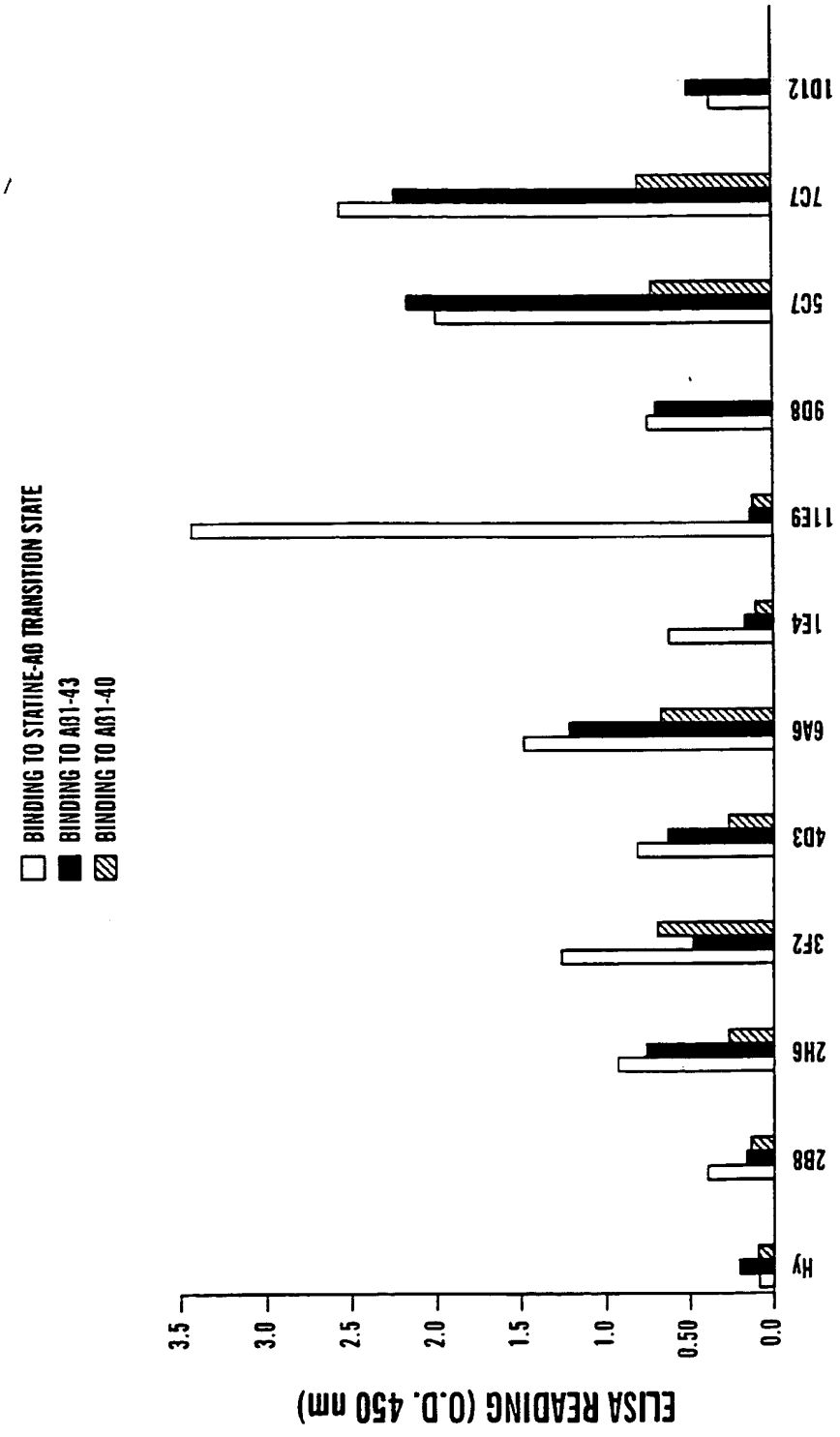

BINDING TO STATINE-AD TRANSITION STATE
BINDING TO AB1-43
BINDING TO AB1-40
ELISA READING (O.D. 450 nm)
3.5
3.0
2.5
2.0
1.5
1.0
0.50
0.0
HY
2B8
2H6
3F2
4D3
6A6
1E4
11E9
9D8
5C7
7C7
10I2
MONOCLONAL ANTIBODY

FIG. 14

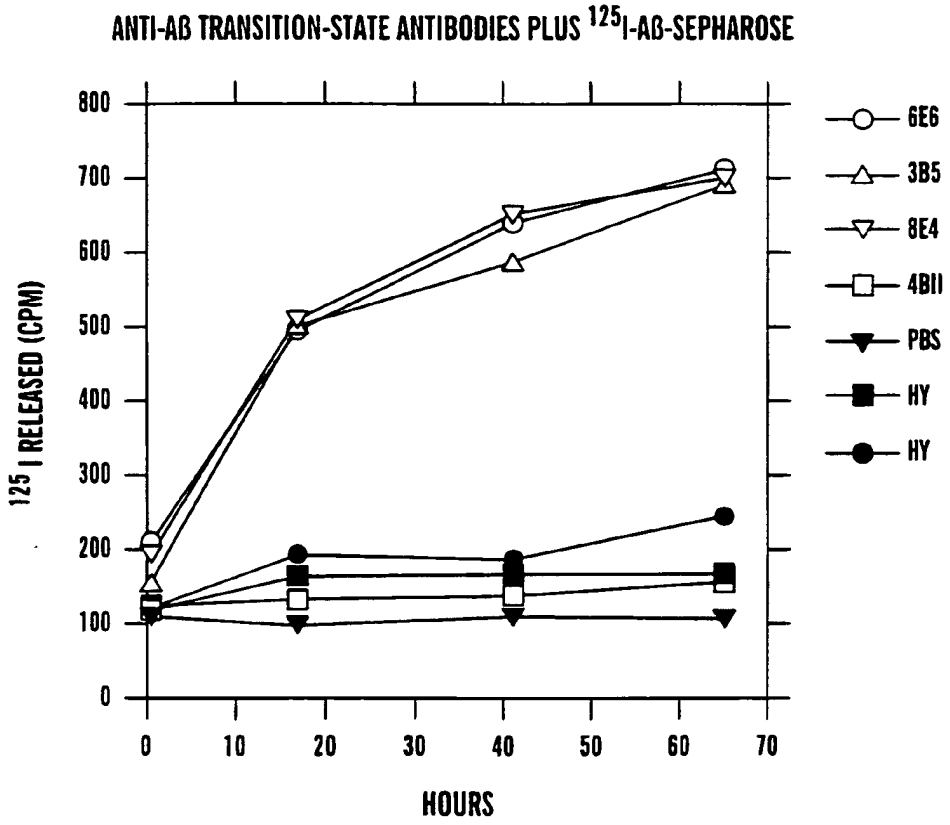

ANTI-Aβ TRANSITION-STATE ANTIBODIES PLUS 125I-Aβ-SEPHAROSE
125I RELEASED (CPM)
800
700
600
500
400
300
200
100
0
0
10
20
30
40
50
60
70
HOURS
6E6
3B5
8E4
4BII
PBS
HY
HY

FIG. 15

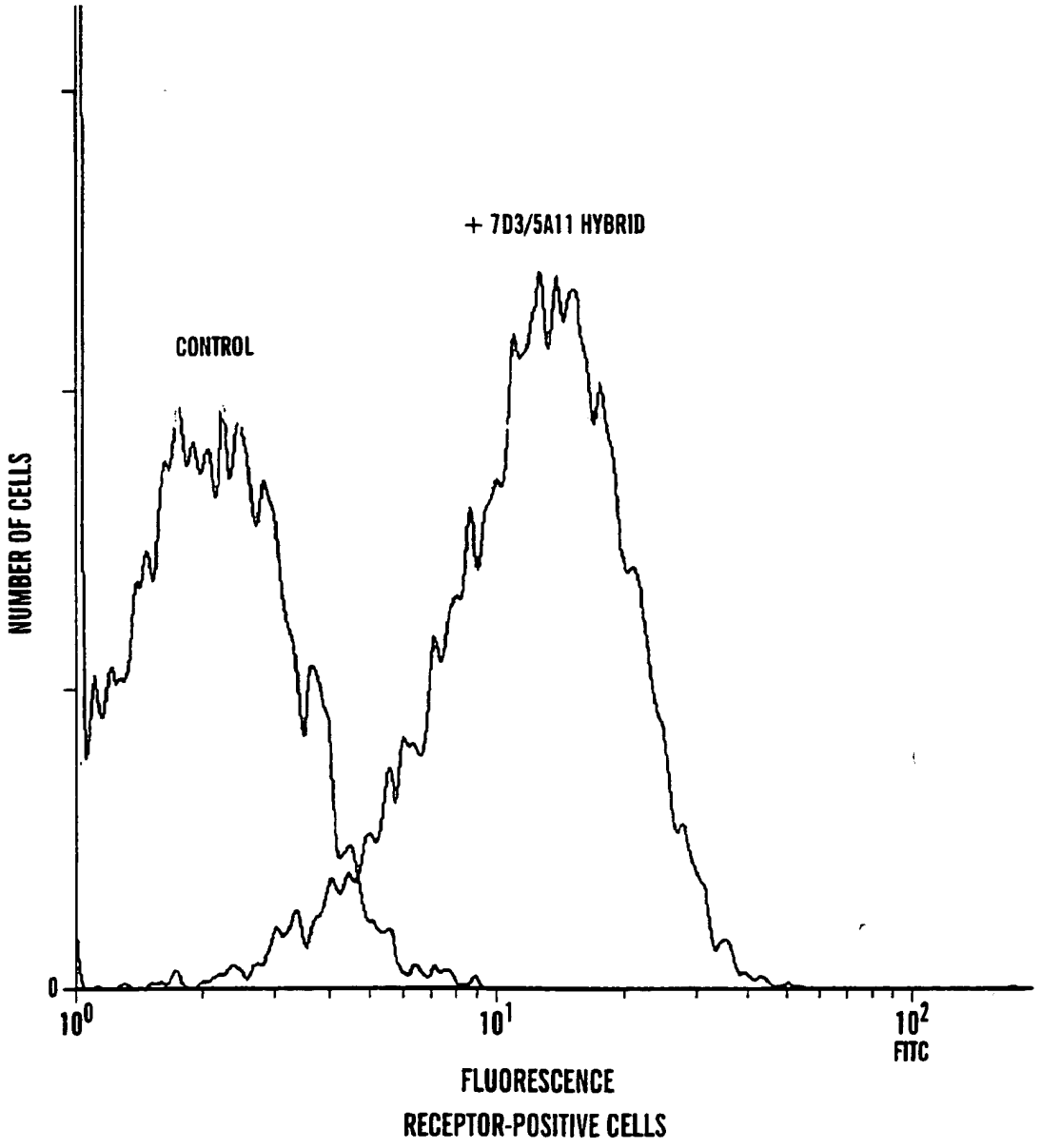

+ 7D3/5A11 HYBRID
CONTROL
NUMBER OF CELLS
0
10^0
10^1
10^2
FITC
FLUORESCENCE
RECEPTOR-POSITIVE CELLS

FIG. 16

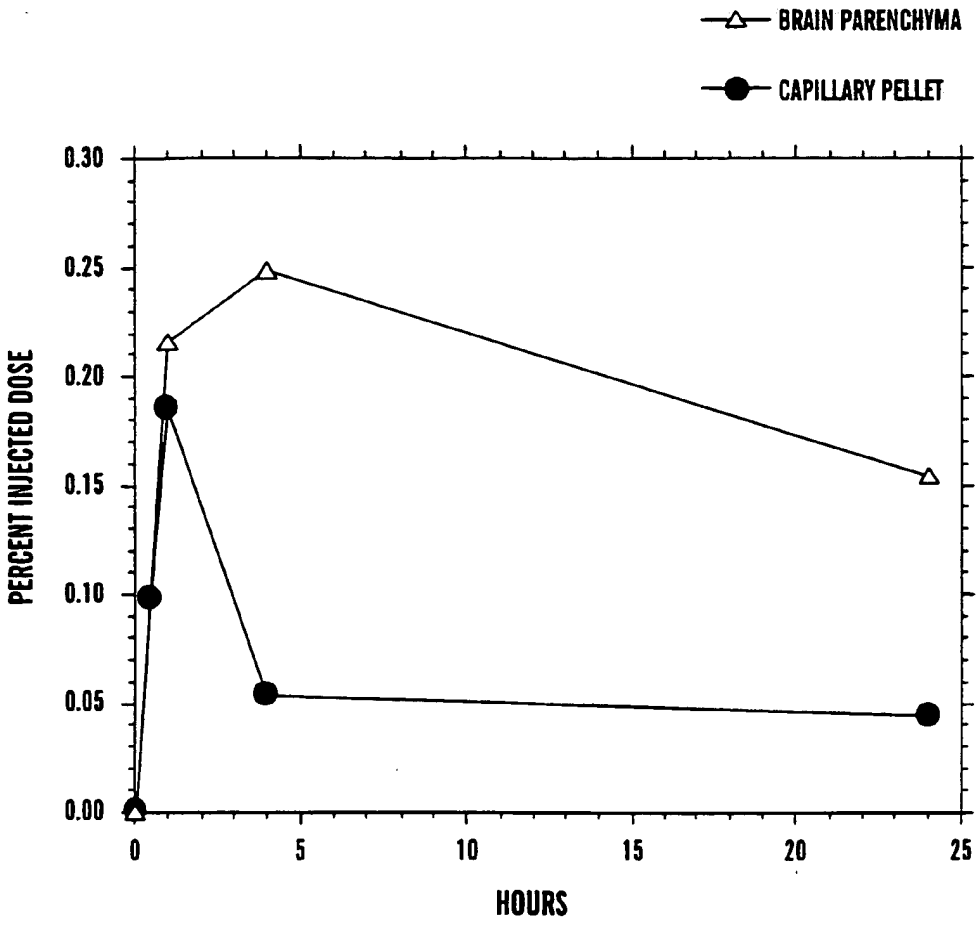

BRAIN PARENCHYMA
CAPILLARY PELLET
PERCENT INJECTED DOSE
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
5
10
15
20
25
HOURS

FIG. 17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/16551

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12N 9/00
US CL :435/188.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/188.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAPLUS & EAST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99/06066 (S. TRASCIATTI, et al.) 11 February 1999, see entire document.	1-6
A	US 5,439,812 (BENKOVIC, et al.) 08 AUGUST 1995,see entire document.	1-10, 33-36
A,P	US 6,043,069 (KOENTGEN, et al.) 28 MARCH 2000, column 15, lines 17-32.	1-10, 33-36

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 12 OCTOBER 2000	Date of mailing of the international search report 17 NOV 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT	Authorized officer Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/16551

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-10, 33-36

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/16551

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-10 and 33-36, drawn to a catalytic antibody which hydrolyzes β-amyloid, a vectorized antibody with the ability to cross the blood barrier and hydrolyze β-amyloid and a method for generating antibodies.

Group II, claims 11-12, drawn to a method for sequestering free β-amyloid in the bloodstream.

Group III, claims 13-22 and 27-32, drawn to a method for reducing levels of β-amyloid in the brain and disaggregating amyloid plaques in the brain.

Group IV, claims 23-24, drawn to a method for preventing the formation of amyloid plaques in the brain.

Group V, claims 25-26, drawn to a method for reducing levels of circulating β-amyloid in an animal.

The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I is drawn to a catalytic antibody and a method of generating antibodies. Groups II-V involve various uses for the catalytic antibody and each use is separate and distinct from the others.

The results of AN1792 or AN1528 treatment with various adjuvants, or thimerosal on cortical amyloid burden in 12-month old mice determined by ELISA are shown in Fig. 15. In PBS control PDAPP mice, the median level of total Aβ in the cortex at 12 months was 1,817 ng/g. Notably reduced levels of Aβ were observed in mice treated with AN1792 plus CFA/IFA, AN1792 plus alum, AN1792 plus MPL and QS-21 plus AN1792. The reduction reached statistical significance ($p<0.05$) only for AN1792 plus CFA/IFA. However, as shown in Examples I and III, the effects of immunization in reducing Aβ levels become substantially greater in 15 month and 18 month old mice. Thus, it is expected that at least the AN1792 plus alum, AN1792 plus MPL and AN1792 plus QS-21 compositions will achieve statistical significance in treatment of older mice. By contrast, the AN1792 plus the preservative thimerosal showed a median level of Aβ about the same as that in the PBS treated mice. Similar results were obtained when cortical levels of Aβ42 were compared. The median level of Aβ42 in PBS controls was 1624 ng/g. Notably reduced median levels of 403, 1149, 620 and 714 were observed in the mice treated with AN1792 plus CFA/IFA, AN1792 plus alum, AN1792 plus MPL and AN1792 plus QS-21 respectively, with the reduction achieving statistical significance ($p=0.05$) for the AN1792 CFA/IFA treatment group. The median level in the AN1792 thimerosal treated mice was 1619 ng/g Aβ42.

A further therapeutic adjuvant/immunogen efficacy study was performed in 9 - 10.5 month old male and female heterozygous PDAPP transgenic mice. The duration of the study was 25 weeks with 29–40 animals per treatment group; therefore the animals were 15 – 16.5 months old at termination.. The treatment groups are identified in Table 10 below.

	Adjuvant	Immunogen	Dilution Buffer	Administration
Group 1:	MPL-SE	AN1792-GCS (75 µg)	PBS	SC (250 µl)
Group 2:	ISA 51	AN1792-GCS (75 µg)	PBS	IP (400 µl)
Group 3:	QS21	AN1792-GCS (75 µg)	PBS	SC (250 µl)
Group 4:	QS21 abbrev.	AN1792-GCS (75 µg)	PBS	SC (250 µl)
Group 5:	PBS	------	------	SC (250 µl)

Table 10 abbreviations: MAP – multi-antigenic peptide; TT – tetanus toxoid t-cell epitope (830-844); SQ – subcutaneous; IP – intraperitoneally; PBS – phosphate, buffered saline; ISA-51 is a commercially available adjuvant similar to IFA; GCS is a

glycine/citrate/sucrose formulation, MPL-SE is MPL in a stabilized water and oil emulsion.

The immunization schedule was identical for all of the treatment groups except for Group 3, the QS21/AN1792 abbreviated schedule group. The mice were injected on weeks 0, 2, 4, 8, 12, 16, 20, 24, with bleeds on weeks 3, 5, 9, 13, 17, 21 and 25. Groups 1, 2, received eight injections and Group 3 received four injections during the 25-week period of the study. Group 4, the QS21/AN1792 abbreviated schedule, received injections on weeks 0, 2, 4, and 8 only. This group was not injected for the remainder of the study, although they were bled on the same bleed schedule as the rest of the study to follow titer decay. Groups 3 and 5, QS21/AN1792 and PBS respectively, served as the positive and negative controls for this study.

The titers were determined by the anti-Aß antibody titer assay.

Group 1, the MPL-SE/AN1792 group, raised a peak geometric mean titer (GMT) of 17,100 at 9 weeks falling to a GMT of 10,000 at 25 weeks. Initially, the MPL-SE titers rose at a somewhat higher rate than the QS21/AN1792 control group (Group 4).

Group 2, the ISA 51/AN1792 group, produced high titers throughout the study reaching a GMT of over 100,000 for the last 9 weeks of the study.

Group 3, the QS21/AN1792 control group, reached its peak titer at 17 weeks with a GMT of 16,000. The titer then fell over the next 8 weeks to finish with a GMT of 8,700. One animal in this group failed to raise a titer over the entire course of the experiment.

Group 4, the QS21/AN1792 abbreviated injection schedule group, reached a peak titer of 7,300 at 13 weeks, five weeks after its final injection. The titer then fell to a GMT of 2,100 at the final bleed (25 weeks). As in the control group, one animal failed to raise a detectable titer, while another animal lost all titer by the end of the decay period.

Group 5, the PBS alone group, had no titers.

To evaluate the cortical Aß levels, total Aß and Aß1-42 were measured by ELISA. Briefly, one brain hemisphere was dissected for cortical, hippocampal, and cerebellar tissue followed by homogenization in 5M guanidine buffer and assayed for brain Aß. The cortical total Aß and Aß42 results are similar. A Mann-Whitney statistical analysis was performed to determine significance between the groups with a p value of 0.05 indicating a significant change in Aß.

All treatment groups significantly lowered total Aß levels as compared to the PBS control group (see Table 11). The MPL-SE/AN1792 group, showed the greatest change in Aß, and it is significantly better than the other treatment groups. The QS21/AN1792 abbreviated group, was similar in its overall change of Aß to the QS21 control group that received all eight injections. The Aß levels in the ISA 51/AN1792 group, were similarly lowered compared to the CFA/IFA:MAP(Aß1-7) group.

Table 11 Cortical Aß levels

	PBS	MPL-SE	ISA	QS-21	QS-21 (4)
MEDIAN (ng/g tissue)	7,335	1,236	3,026	2,389	2,996
RANGE (ng/g tissue)	550 – 18,358	70 – 3,977	23 – 9,777	210 – 11,167	24 – 16,834
p value	----	< 0.0001	< 0.0001	< 0.0001	< 0.0001
N	38	29	36	34	40

In conclusion, MPL-SE, ISA-51 and QS21 adjuvants combined with AN1792 are effective in inducing a sufficient immune response significantly to retard Aß deposition in the cortex.

X. Toxicity Analysis

Tissues were collected for histopathologic examination at the termination of studies described in Examples 2, 3 and 7. In addition, hematology and clinical chemistry were performed on terminal blood samples from Examples 3 and 7. Most of the major organs were evaluated, including brain, pulmonary, lymphoid, gastrointestinal, liver, kidney, adrenal and gonads. Although sporadic lesions were observed in the study animals, there were no obvious differences, either in tissues affected or lesion severity, between AN1792 treated and untreated animals. There were no unique histopathological lesions noted in AN-1528-immunized animals compared to PBS-treated or untreated animals. There were also no differences in the clinical chemistry profile between adjuvant groups and the PBS treated animals in Example 7. Although there were significant increases in several of the hematology parameters between animals treated with AN1792 and Freund's adjuvant in Example 7 relative to PBS treated animals, these

type of effects are expected from Freund's adjuvant treatment and the accompanying peritonitis and do not indicate any adverse effects from AN1792 treatment. Although not part of the toxicological evaluation, PDAPP mouse brain pathology was extensively examined as part of the efficacy endpoints. No sign of treatment related adverse effect on brain morphology was noted in any of the studies. These results indicate that AN1792 treatment is well tolerated and at least substantially free of side effects.

XI. Therapeutic Treatment with Anti-Aβ antibodies

This examples tests the capacity of various monoclonal and polyclonal antibodies to Aβ to inhibit accumulation of Aβ in the brain of heterozygotic transgenic mice.

1. Study Design

Sixty male and female, heterozygous PDAPP transgenic mice, 8.5 to 10.5 months of age were obtained from Charles River Laboratory. The mice were sorted into six groups to be treated with various antibodies directed to Aβ. Animals were distributed to match the gender, age, parentage and source of the animals within the groups as closely as possible. As shown in Table 10, the antibodies included four murine Aβ-specific monoclonal antibodies, 2H3 (directed to Aβ residues 1-12), 10D5 (directed to Aβ residues 1-16), 266 (directed to Aβ residues 13-28 and binds to monomeric but not to aggregated AN1792), 21F12 (directed to Aβ residues 33-42). A fifth group was treated with an Aβ-specific polyclonal antibody fraction (raised by immunization with aggregated AN1792). The negative control group received the diluent, PBS, alone without antibody.

The monoclonal antibodies were injected at a dose of about 10 mg/kg (assuming that the mice weighed 50 g). Injections were administered intraperitoneally every seven days on average to maintain anti-Aβ titers above 1000. Although lower titers were measured for mAb 266 since it does not bind well to the aggregated AN1792 used as the capture antigen in the assay, the same dosing schedule was maintained for this group. The group receiving monoclonal antibody 2H3 was discontinued within the first three weeks since the antibody was cleared too rapidly in vivo. Animals were bled prior to each dosing for the measurement of antibody titers. Treatment was continued over a

six-month period for a total of 196 days. Animals were euthanized one week after the final dose.

Table 12

EXPERIMENTAL DESIGN

Treatment Group	N[a]	Treatment Antibody	Antibody Specificity	Antibody Isotype
1	9	none (PBS alone)	NA[b]	NA
2	10	Polyclonal	Aβ1-42	mixed
3	0	mAb[c] 2H3	Aβ1-12	IgG1
4	8	mAb 10D5	Aβ1-16	IgG1
5	6	mAb 266	Aβ13-28	IgG1
6	8	mAb 21F12	Aβ33-42	IgG2a

Footnotes

a. Number of mice in group at termination of the experiment. All groups started with 10 animals per group.

b. NA: not applicable

c. mAb: monoclonal antibody

2. Materials and Methods

a. Preparation of the Antibodies

The anti-Aβ polyclonal antibody was prepared from blood collected from two groups of animals. The first group consisted of 100 female Swiss Webster mice, 6 to 8 weeks of age. They were immunized on days 0, 15, and 29 with 100 μg of AN1792 combined with CFA/IFA. A fourth injection was given on day 36 with one-half the dose of AN1792. Animals were exsanguinated upon sacrifice at day 42, serum was prepared and the sera were pooled to create a total of 64 ml. The second group consisted of 24 female mice isogenic with the PDAPP mice but nontransgenic for the human APP gene, 6 to 9 weeks of age. They were immunized on days 0, 14, 28 and 56 with 100 μg of AN1792 combined with CFA/IFA. These animals were also exsanguinated upon sacrifice at day 63, serum was prepared and pooled for a total of 14 ml. The two lots of sera were pooled. The antibody fraction was purified using two sequential rounds of

precipitation with 50% saturated ammonium sulfate. The final precipitate was dialyzed against PBS and tested for endotoxin. The level of endotoxin was less than 1 EU/mg.

The anti-Aβ monoclonal antibodies were prepared from ascites fluid. The fluid was first delipidated by the addition of concentrated sodium dextran sulfate to ice-cold ascites fluid by stirring on ice to a reach a final concentration of 0.238%. Concentrated $CaCl_2$ was then added with stirring to reach a final concentration of 64mM. This solution was centrifuged at 10,000 x g and the pellet was discarded. The supernatant was stirred on ice with an equal volume of saturated ammonium sulfate added dropwise. The solution was centrifuged again at 10,000 x g and the supernatant was discarded. The pellet was resuspended and dialyzed against 20 mM Tris-HCl , 0.4 M NaCl, pH 7.5. This fraction was applied to a Pharmacia FPLC Sepharose Q Column and eluted with a reverse gradient from 0.4 M to 0.275 M NaCl in 20 mM Tris-HCl, pH 7.5.

The antibody peak was identified by absorbance at 280 nm and appropriate fractions were pooled. The purified antibody preparation was characterized by measuring the protein concentration using the BCA method and the purity using SDS-PAGE. The pool was also tested for endotoxin. The level of endotoxin was less than 1 EU/mg. titers, titers less than 100 were arbitrarily assigned a titer value of 25.

3. Aβ and APP Levels in the Brain:

Following about six months of treatment with the various anti-Aβ antibody preparations, brains were removed from the animals following saline perfusion. One hemisphere was prepared for immunohistochemical analysis and the second was used for the quantitation of Aβ and APP levels. To measure the concentrations of various forms of beta amyloid peptide and amyloid precursor protein (APP), the hemisphere was dissected and homogenates of the hippocampal, cortical, and cerebellar regions were prepared in 5M guanidine. These were serially diluted and the level of amyloid peptide or APP was quantitated by comparison to a series of dilutions of standards of Aβ peptide or APP of known concentrations in an ELISA format.

The levels of total Aβ and of Aβ1-42 measured by ELISA in homogenates of the cortex, and the hippocampus and the level of total Aβ in the cerebellum are shown in Tables 11, 12, and 13, respectively. The median concentration of total Aβ for the control group, inoculated with PBS, was 3.6-fold higher in the hippocampus than in the cortex (median of 63,389 ng/g hippocampal tissue compared to 17,818 ng/g for the

cortex). The median level in the cerebellum of the control group (30.6 ng/g tissue) was more than 2,000-fold lower than in the hippocampus. These levels are similar to those that we have previously reported for heterozygous PDAPP transgenic mice of this age (Johnson-Wood et al., 1997).

For the cortex, one treatment group had a median Aβ level, measured as Aβ1-42, which differed significantly from that of the control group ($p < 0.05$), those animals receiving the polyclonal anti-Aβ antibody as shown in Table 13. The median level of Aβ1-42 was reduced by 65%, compared to the control for this treatment group. The median levels of Aβ1-42 were also significantly reduced by 55% compared to the control in one additional treatment group, those animals dosed with the mAb 10D5 ($p = 0.0433$).

Table 13

CORTEX

Treatment Group	N[a]	Medians						Means	
		Total Aβ			Aβ42			Total Aβ	Aβ42
		LISA value[b]	P value[c]	% Change	ELISA value	P value	% Change	ELISA value	ELISA value
PBS	9	17818	NA[d]	NA	13802	NA	NA	16150+/-7456[e]	12621+/-5738
Polyclonal anti-Aβ42	10	6160	0.0055	-65	4892	0.0071	-65	5912+/-4492	4454+/-3347
mAb 10D5	8	7915	0.1019	-56	6214	0.0433	-55	9695+/-6929	6943+/-3351
mAb 266	6	9144	0.1255	-49	8481	0.1255	-39	9204+/-9293	7489+/-6921
mAb 21F12	8	15158	0.2898	-15	13578	0.7003	-2	12481+/-7082	11005+/-6324

Footnotes:

a. Number of animals per group at the end of the experiment
b. ng/g tissue
c. Mann Whitney analysis
d. NA: not applicable
e. Standard Deviation

In the hippocampus, the median percent reduction of total Aβ associated with treatment with polyclonal anti-Aβ antibody (50%, p = 0.0055) was not as great as that observed in the cortex (65%) (Table 14). However, the absolute magnitude of the reduction was almost 3-fold greater in the hippocampus than in the cortex, a net reduction of 31,683 ng/g tissue in the hippocampus versus 11,658 ng/g tissue in the cortex. When measured as the level of the more amyloidogenic form of Aβ, Aβ1-42, rather than as total Aβ, the reduction achieved with the polyclonal antibody was significant (p = 0.0025). The median levels in groups treated with the mAbs 10D5 and 266 were reduced by 33% and 21%, respectively.

Table 14

HIPPOCAMPUS									
Treatment Group	N[a]	Medians						Means	
		Total Aβ			Aβ42			Total Aβ	Aβ42
		ELISA value[b]	P value[c]	% Change	ELISA value	P value	% Change	ELISA value	ELISA value
PBS	9	63389	NA[d]	NA	54429	NA	NA	58351+/-13308[e]	52801+/-14701
Polyclonal anti-Aβ42	10	31706	0.0055	-50	27127	0.0025	-50	30058+/-22454	24853+/-18262
mAb 10D5	8	46779	0.0675	-26	36290	0.0543	-33	44581+/-18632	36465+/-17146
mAb 266	6	48689	0.0990	-23	43034	0.0990	-21	36419+/-27304	32919+/-25372
mAb 21F12	8	51563	0.7728	-19	47961	0.8099	-12	57327+/-28927	50305+/-23927

Footnotes:

a. Number of animals per group at the end of the experiment

b. ng/g tissue

c. Mann Whitney analysis

d. NA: not applicable

e. Standard Deviation

Total Aβ was also measured in the cerebellum (Table 15). Those groups dosed with the polyclonal anti-Aβ and the 266 antibody showed significant reductions of the levels of total Aβ (43% and 46%, $p = 0.0033$ and $p = 0.0184$, respectively) and that group treated with 10D5 had a near significant reduction (29%, $p = 0.0675$).

Table 15

CEREBELLUM					
Treatment Group	N[a]	Medians			Means
		Total Aβ			Total Aβ
		ELISA value[b]	P value[c]	% Change	ELISA value
PBS	9	30.64	NA[d]	NA	40.00+/-31.89[e]
Polyclonal anti-Aβ42	10	17.61	0.0033	-43	18.15+/-4.36
mAb 10D5	8	21.68	0.0675	-29	27.29+/-19.43
mAb 266	6	16.59	0.0184	-46	19.59+/-6.59
mAb 21F12	8	29.80	>0.9999	-3	32.88+/-9.90

Footnotes:

a. Number of animals per group at the end of the experiment

b. ng/g tissue

c. Mann Whitney analysis

d. NA: not applicable

e. Standard Deviation

APP concentration was also determined by ELISA in the cortex and cerebellum from antibody-treated and control, PBS-treated mice. Two different APP assays were utilized. The first, designated APP-α/FL, recognizes both APP-alpha (α, the secreted form of APP which has been cleaved within the Aβ sequence), and full-length forms (FL) of APP, while the second recognizes only APP-α. In contrast to the treatment-associated diminution of Aβ in a subset of treatment groups, the levels of APP were virtually unchanged in all of the treated compared to the control animals. These results indicate that the immunizations with Aβ antibodies deplete Aβ without depleting APP.

In summary, Aβ levels were significantly reduced in the cortex, hippocampus and cerebellum in animals treated with the polyclonal antibody raised against AN1792. To a lesser extent monoclonal antibodies to the amino terminal region of Aβ1-42, specifically amino acids 1-16 and 13-28 also showed significant treatment effects.

4. Histochemical Analyses:

The morphology of Aß-immunoreactive plaques in subsets of brains from mice in the PBS, polyclonal Aß42, 21F12, 266 and 10D5 treatment groups was qualitatively compared to that of previous studies in which standard immunization procedures with Aß42 were followed.

The largest alteration in both the extent and appearance of amyloid plaques occurred in the animals immunized with the polyclonal Aß42 antibody. The reduction of amyloid load, eroded plaque morphology and cell-associated Aß immunoreactivity closely resembled effects produced by the standard immunization procedure. These observations support the ELISA results in which significant reductions in both total Aß and Aß42 were achieved by administration of the polyclonal Aß42 antibody.

In similar qualitative evaluations, amyloid plaques in the 10D5 group were also reduced in number and appearance, with some evidence of cell-associated Aß immunoreactivity. Relative to control-treated animals, the polyclonal Ig fraction against Aß and one of the monoclonal antibodies (10D5) reduced plaque burden by 93% and 81%, respectively ($p<0.005$). 21F12 appeared to have a relatively modest effect on plaque burden. Micrographs of brain after treatment with pab$Aß_{1-42}$ show diffuse deposits and absence of many of the larger compacted plaques in the pab$Aß_{1-42}$ treated group relative to control treated animals.

5. Measurement of Antibody Titers:

A subset of three randomly chosen mice from each group were bled just prior to each intraperitoneal inoculation, for a total of 30 bleeds. Antibody titers were measured as Aβ1-42-binding antibody using a sandwich ELISA with plastic multi-well plates coated with Aβ1-42 as described in detail in the General Materials and Methods. Mean titers for each bleed are shown in Figures 16-18 for the polyclonal antibody and the monoclonals 10D5 and 21F12, respectively. Titers averaged about 1000 over this time period for the polyclonal

antibody preparation and were slightly above this level for the 10D5- and 21F12-treated animals.

6. Lymphoproliferative Responses:

Aβ-dependent lymphoproliferation was measured using spleen cells harvested eight days following the final antibody infusion. Freshly harvested cells, 10^5 per well, were cultured for 5 days in the presence of Aβ1-40 at a concentration of 5 μM for stimulation. As a positive control, additional cells were cultured with the T cell mitogen, PHA, and, as a negative control, cells were cultured without added peptide.

Splenocytes from aged PDAPP mice passively immunized with various anti-Aβ antibodies were stimulated *in vitro* with AN1792 and proliferative and cytokine responses were measured. The purpose of these assays was to determine if passive immunization facilitated antigen presentation, and thus priming of T cell responses specific for AN1792. No AN1792-specific proliferative or cytokine responses were observed in mice passively immunized with the anti-Aβ antibodies.

XII: FURTHER STUDY OF PASSIVE IMMUNIZATION

In a second study, treatment with 10D5 was repeated and two additional anti-Aß antibodies were tested, monoclonals 3D6 ($Aß_{1-5}$) and 16C11 ($Aß_{33-42}$). Control groups received either PBS or an irrelevant isotype-matched antibody (TM2a). The mice were older (11.5-12 month old heterozygotes) than in the previous study, otherwise the experimental design was the same. Once again, after six months of treatment, 10D5 reduced plaque burden by greater than 80% relative to either the PBS or isotype-matched antibody controls ($p=0.003$). One of the other antibodies against Aß, 3D6, was equally effective, producing an 86% reduction ($p=0.003$). In contrast, the third antibody against the peptide, 16C11, failed to have any effect on plaque burden. Similar findings were obtained with $Aß_{42}$ ELISA measurements. These results demonstrate that an antibody response against Aß peptide, in the absence of T cell immunity, is sufficient to decrease amyloid deposition in PDAPP mice, but that not all anti-Aß antibodies are efficacious. Antibodies directed to epitopes comprising amino acids 1-5 or 3-7 of Aβ are particularly efficacious.

In summary, we have shown that passively administered antibodies against Aß reduced the extent of plaque deposition in a mouse model of Alzheimer's disease. When held

at modest serum concentrations (25–70 μg/ml), the antibodies gained access to the CNS at levels sufficient to decorate ß-amyloid plaques. Antibody entry into the CNS was not due to abnormal leakage of the blood-brain barrier since there was no increase in vascular permeability as measured by Evans Blue in PDAPP mice. In addition, the concentration of antibody in the brain parenchyma of aged PDAPP mice was the same as in non-transgenic mice, representing 0.1% of the antibody concentration in serum (regardless of isotype).

XIII: MONITORING OF ANTIBODY BINDING

To determine whether antibodies against Aß could be acting directly within the CNS, brains taken from saline-perfused mice at the end of the Example XII, were examined for the presence of the peripherally-administered antibodies. Unfixed cryostat brain sections were exposed to a fluorescent reagent against mouse immunoglobulin (goat anti-mouse IgG-Cy3). Plaques within brains of the 10D5 and 3D6 groups were strongly decorated with antibody, while there was no staining in the 16C11 group. To reveal the full extent of plaque deposition, serial sections of each brain were first immunoreacted with an anti-Aß antibody, and then with the secondary reagent. 10D5 and 3D6, following peripheral administration, gained access to most plaques within the CNS. The plaque burden was greatly reduced in these treatment groups compared to the 16C11 group. These data indicate that peripherally administered antibodies can enter the CNS where they can directly trigger amyloid clearance. It is likely that 16C11 also had access to the plaques but was unable to bind.

XIV: EX VIVO SCREENING ASSAY FOR ACTIVITY OF AN ANTIBODY AGAINST AMYLOID DEPOSITS

To examine the effect of antibodies on plaque clearance, we established an *ex vivo* assay in which primary microglial cells were cultured with unfixed cryostat sections of either PDAPP mouse or human AD brains. Microglial cells were obtained from the cerebral cortices of neonate DBA/2N mice (1-3 days). The cortices were mechanically dissociated in HBSS$^-$ (Hanks' Balanced Salt Solution, Sigma) with 50 µg/ml DNase I (Sigma). The dissociated cells were filtered with a 100 µm cell strainer (Falcon), and centrifuged at 1000 rpm for 5 minutes. The pellet was resuspended in growth medium (high glucose DMEM, 10%FBS, 25ng/ml rmGM-CSF), and the cells were plated at a density of 2 brains per T-75 plastic culture flask. After 7-9 days, the flasks were rotated on an orbital shaker at 200 rpm for 2h at 37°C. The cell suspension was centrifuged at 1000rpm and resuspended in the assay medium.

10-µm cryostat sections of PDAPP mouse or human AD brains (post-mortem interval < 3hr) were thaw mounted onto poly-lysine coated round glass coverslips and placed in wells of 24-well tissue culture plates. The coverslips were washed twice with assay medium consisting of H-SFM (Hybridoma-serum free medium, Gibco BRL) with 1% FBS, glutamine, penicillin/streptomycin, and 5ng/ml rmGM-CSF (R&D). Control or anti-Aß antibodies were added at a 2x concentration (5 µg/ml final) for 1 hour. The microglial cells were then seeded at a density of 0.8x 10^6 cells/ml assay medium. The cultures were maintained in a humidified incubator (37°C, 5%CO_2) for 24hr or more. At the end of the incubation, the cultures were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton-X100. The sections were stained with biotinylated 3D6 followed by a streptavidin / Cy3 conjugate (Jackson ImmunoResearch). The exogenous microglial cells were visualized by a nuclear stain (DAPI). The cultures were observed with an inverted fluorescent microscope (Nikon, TE300) and photomicrographs were taken with a SPOT digital camera using SPOT software (Diagnostic instruments). For Western blot analysis, the cultures were extracted in 8M urea, diluted 1:1 in reducing tricine sample buffer and loaded onto a 16% tricine gel (Novex). After transfer onto immobilon, blots were exposed to 5 µg/ml of the pabAß42 followed by an HRP-conjugated anti-mouse antibody, and developed with ECL (Amersham)

When the assay was performed with PDAPP brain sections in the presence of 16C11 (one of the antibodies against Aß that was not efficacious *in vivo*), ß-amyloid plaques remained intact and no phagocytosis was observed. In contrast, when adjacent sections were

cultured in the presence of 10D5, the amyloid deposits were largely gone and the microglial cells showed numerous phagocytic vesicles containing Aß. Identical results were obtained with AD brain sections; 10D5 induced phagocytosis of AD plaques, while 16C11 was ineffective. In addition, the assay provided comparable results when performed with either mouse or human microglial cells, and with mouse, rabbit, or primate antibodies against Aß.

Table 16 shows whether binding and/or phagocytosis was obtained for several different antibody binding specificities. It can be seen that antibodies binding to epitopes within aa 1-7 both bind and clear amyloid deposits, whereas antibodies binding to epitopes within amino acids 4-10 bind without clearing amyloid deposits. Antibodies binding to epitopes C-terminal to residue 10 neither bind nor clear amyloid deposits.

Table 16: Analysis of Epitope Specificity

	Antibody		Staining	Phagocytosis
	epitope	isotype		
N-Term				
mab				
3D6	1-5	IgG2b	+	+
10D5	3-6	IgG1	+	+
22C8	3-7	IgG2a	+	+
6E10	5-10	IgG1	+	-
14A8	4-10	rat IgG1	+	-
13-28				
18G11	10-18	rat IgG1	-	-
266	16-24	IgG1	-	-
22D12	18-21	IgG2b	-	-
C-Term				
2G3	-40	IgG1	-	-
16C11	-40/-42	IgG1	-	-
21F12	-42	IgG2a	-	-
Immune serum				
rabbit (CFA)	1-6		+	+
mouse (CFA)	3-7		+	+
mouse (QS-21)	3-7		+	+
monkey (QS-21)	1-5		+	+
mouse (MAP1-7)			+	+

Table 17 shows results obtained with several antibodies against Aß, comparing their abilities to induce phagocytosis in the *ex vivo* assay and to reduce *in vivo* plaque burden in passive transfer studies. Although 16C11 and 21F12 bound to aggregated synthetic Aß peptide with high avidity, these antibodies were unable to react with ß-amyloid plaques in unfixed brain sections, could not trigger phagocytosis in the *ex vivo* assay, and were not efficacious *in vivo*. 10D5, 3D6, and the polyclonal antibody against Aß were active by all three measures. The 22C8 antibody binds more strongly to an analog form of natural

Aß in which aspartic acid at positions 1 and 7 is replaced with iso-aspartic acid. These results show that efficacy *in vivo* is due to direct antibody mediated clearance of the plaques within the CNS, and that the *ex vivo* assay is predictive of *in vivo* efficacy.

The same assay has been used to test clearing of an antibody against a fragment of synuclein referred to as NAC. Synuclein has been shown to be an amyloid plaque-associated protein. An antibody to NAC was contacted with a brain tissue sample containing amyloid plaques, an microglial cells, as before. Rabbit serum was used as a control. Subsequent monitoring showed a marked reduction in the number and size of plaques indicative of clearing activity of the antibody.

Table 17 The *ex vivo* assay as predictor of *in vivo* efficacy.

Antibody	Isotype	Avidity for aggregated Aß (pM)	Binding to ß-amyloid plaques	*Ex vivo* efficacy	*In vivo* efficacy
monoclonal					
3D6	IgG2b	470	+	+	+
10D5	IgG1	43	+	+	+
16C11	IgG1	90	-	-	-
21F12	IgG2a	500	-	-	-
TM2a	IgG1	-	-	-	-
polyclonal					
1-42	mix	600	+	+	+

Confocal microscopy was used to confirm that Aß was internalized during the course of the *ex vivo* assay. In the presence of control antibodies, the exogenous microglial cells remained in a confocal plane above the tissue, there were no phagocytic vesicles containing Aß, and the plaques remained intact within the section. In the presence of 10D5, nearly all plaque material was contained in vesicles within the exogenous microglial cells. To determine the fate of the internalized peptide, 10D5 treated cultures were extracted with 8M urea at various time-points, and examined by Western blot analysis. At the one hour time point, when no phagocytosis had yet occurred, reaction with a polyclonal antibody against Aß revealed a strong 4 kD band (corresponding to the Aß peptide). Aß immunoreactivity

decreased at day 1 and was absent by day 3. Thus, antibody-mediated phagocytosis of Aß leads to its degradation.

To determine if phagocytosis in the *ex vivo* assay was Fc-mediated, F(ab')2 fragments of the anti-Aß antibody 3D6 were prepared. Although the F(ab')2 fragments retained their full ability to react with plaques, they were unable to trigger phagocytosis by microglial cells. In addition, phagocytosis with the whole antibody could be blocked by a reagent against murine Fc receptors (anti-CD16/32). These data indicate that *in vivo* clearance of Aß occurs through Fc-receptor mediated phagocytosis.

XV: PASSAGE OF ANTIBODIES THROUGH BLOOD BRAIN BARRIER

This example determines the concentration of antibody delivered to the brain following intravenous injection into a peripheral tissue of either normal or PDAPP mice. PDAPP or control normal mice were perfused with 0.9% NaCl. Brain regions (hippocampus or cortex) were dissected and rapidly frozen. Brain were homogenized in 0.1% triton + protease inhibitors. Immunoglobulin was detected in the extracts by ELISA. Fab'2 Goat Anti-mouse IgG were coated onto an RIA plate as capture reagent. The serum or the brain extracts were incubated for 1hr. The isotypes were detected with anti-mouse IgG1-HRP or IgG2a-HRP or IgG2b-HRP (Caltag). Antibodies, regardless of isotype, were present in the CNS at a concentration that is 1:1000 that found in the blood. For example, when the concentration of IgG1 was three times that of IgG2a in the blood, it was three times IgG2a in the brain as well, both being present at 0.1% of their respective levels in the blood. This result was observed in both transgenic and nontransgenic mice - so the PDAPP does not have a uniquely leak blood brain barrier.

XVI: THERAPEUTIC EFFICACY OF AN Aß PEPTIDE IN MAP CONFIGURATION

A therapeutic adjuvant/immunogen efficacy study was performed in 9 - 10.5 month old male and female heterozygous PDAPP transgenic mice to test the efficacy of a fusion protein comprising Aß1-7 in tetrameric MAP configuration as described above. The duration of the study was 25 weeks with 29 – 40 animals per treatment group; therefore the animals were 15 – 16.5 months old at termination. The methodology used in this study is the same as that in the therapeutic study of different adjuvants in Example VIII above. . The treatment groups are identified in Table 18 below.

Table 18

	Adjuvant	Immunogen	Dilution Buffer	Administration
Group 1:	CFA/IFA	MAP(Aß 1-7:TT) (100 µg)	PBS	IP (400 µl)
Group 2:	QS21	AN1792-GCS (75 µg)	PBS	SC (250 µl)
Group 3:	PBS	------	------	SC (250 µl)

Table abbreviations: MAP – multi-antigenic peptide; TT – tetanus toxoid t-cell epitope (830-844); SC – subcutaneous; IP – intraperitoneally; PBS – phosphate buffered saline; GCS is a glycine/citrate/sucrose formulation.

The immunization schedule was identical for all of the treatment groups. The mice were injected on weeks 0, 2, 4, 8, 12, 16, 20, 24, with bleeds on week 3, 5, 9, 13, 17, 21 and 25. Groups 1, 2, 3, 4, and 6 received eight injections Groups 2 and 3, QS21/AN1792 and PBS respectively, served as the positive and negative controls for this study.

The titers were determined by the anti-Aß antibody titer assay.

Group 1, CFA/IFA:MAP(Aß1-7:TT) group, had low titer levels. The peak GMT reached was only 1,200 at 13 weeks, falling to a GMT of 600 by week 25. There were 3 of the 30 mice that did not raise any titer and another 7 mice that did not exceed a titer of 400 by the end of the study.

Group 2, the QS21/AN1792 control group, reached its peak titer at 17 weeks with a GMT of 16,000. The titer then fell over the next 8 weeks to finish with a GMT of 8,700. One animal in this group failed to raise a titer over the entire course of the experiment.

Group 3, the PBS alone group, had no titers.

Both treatment groups showed a significant lowing in cortical Aß levels as compared to the PBS control group (see Table 19). The CFA/IFA:MAP(Aß1-7) group, significantly lowered Aß as compared to the PBS control group in spite of the relatively low titers of anti-Aß antibodies.

Table 19 Cortical Aß levels

	PBS	MAP	QS-21
MEDIAN (ng/g tissue)	7,335	3,692	2,389
RANGE (ng/g tissue)	550 – 18,358	240 – 10,782	210 – 11,167
p value	----	0.0003	< 0.0001
N	38	30	34

In conclusion, the Aß 1-7MAP immunogen is effective in inducing a sufficient immune response significantly to retard Aß deposition in the cortex.

XVII. EPITOPE MAPPING OF IMMUNOGENIC RESPONSE TO Aβ IN MONKEYS

This example analyzes the response of a primate to immunization with AN1792 (i.e., Aβ1-42). Eleven groups of monkeys (4/sex/group) were immunized with AN1792 (75 or 300 μg/dose) in combination with QS-21 adjuvant (50 or 100 μg/dose) or 5% sterile dextrose in water (D5W, control group). All animals received IM injections on one of three injection schedules as shown in Table 20 for a total of 4, 5 or 8 doses. Serum samples (from 4 monkeys/sex/group) collected on Day 175 of the study and CSF samples (from 3 monkeys/sex/group) collected on Day 176 of the study (at the 6 month necropsy) were evaluated for their ability to bind to Aβ1-40 peptide and APP.

Table 20: Group Assignments and Dose Levels

Group No.	Schedule[a]	# Monkeys (M/F)	AN1792 Dose (μg/dos)	QS-21 Dose (μg/dose)	Dose Route
1[b]	1	4/4	0	0	IM
2	1	4/4	Vehicle[c]	50	IM
3	1	4/4	Vehicle	100	IM
4	1	4/4	75	50	IM
5	1	4/4	300	50	IM
6	1	4/4	75	100	IM
7	1	4/4	300	100	IM
8	2	4/4	75	100	IM
9	2	4/4	300	100	IM
10	3	4/4	75	100	IM
11	3	4/4	300	100	IM

a. Schedule 1, Dose Days 1, 15, 29, 57, 85, 113, 141, 169; Schedule 2, Dose Days 1, 29, 57, 113, 169; Schedule 3, Dose Days 1, 43, 85, 169

b. D5W injection control group

c. Vehicle consists of the glycine/citrate/sucrose buffer which is the excipient for AN1792.

The exact array of linear peptides recognized by the antibodies in the serum samples from animals immunized with AN1792 was determined by an ELISA that measured the binding of these antibodies to overlapping peptides that covered the entire Aβ1-42 sequence. Biotinylated peptides with partial sequences of AN1792 were obtained from Chiron Technologies as 10 amino acid peptides with an overlap of 9 residues and a step of one residue per peptide (synthesis No. 5366, No. 5331 and No. 5814). The first 32 peptides (from the eight amino acid position upstream of the N-terminal of AN1792 down to the twenty-fourth amino acid of AN1792) are biotinylated on the C-terminal with a linker of GGK. The last 10 peptides (repeating the thirty-second peptide from the previous series) are biotinylated on the N-terminal with a linker consisting of EGEG). The lyophilized biotinylated peptides were dissolved at a concentration of 5 mM in DMSO. These peptide stocks were diluted to 5 μM in TTBS (0.05% Tween 20, 25 mM Tris HCl, 137 mM NaCl, 5.1 mM KCl, pH=7.5). 100 μl aliquots of this 5 μM solution were added in duplicate to streptavidin pre-coated 96-well plates (Pierce). Plates were incubated for one hour at room temperature, then washed four times with TTBS. Serum samples were diluted in specimen diluent without azide to normalize titers, and 100 μl was added per well. These plates were incubated one hour at room temperature and then washed four times with TTBS. HRP-

conjugated goat anti-human antibody (Jackson ImmunoResearch) was diluted 1:10,000 in specimen diluent without azide and 100 µl was added per well. The plates were again incubated and washed. To develop the color reaction, TMB (Pierce), was added at 100 µl per well and incubated for 15 min prior to the addition of 30 µl of 2 N H_2SO_4 to stop the reaction. The optical density was measured at 450 nm on a Vmax or Spectramax colorimetric plate reader.

Immunization with AN1792 resulted in the production of antibodies in 100% of the animals in all of the dose groups by Day 175. Mean titers in the groups ranged from 14596 - 56084. There was a trend for titers to be higher within an immunization schedule in the presence of higher antigen and/or higher adjuvant concentration, but no statistically significant differences could be demonstrated due to the high variability in individual animal responses to the immunizations.

Sera which were positive for antibodies to AN1792 were also positive for antibodies to Aβ1-40. Mean titers in the groups ranged from 36867 - 165991, and as for anti-AN1792 titers, showed no statistically significant differences between groups at Day 175. Binding to AN1792 showed a highly positive correlation (Spearman r = 0.8671) with binding to Aβ1-40.

Of the 48 monkeys immunized on various schedules with AN1792, 33 yielded CSF samples of adequate volume and quality for analysis. Thirty-two (97%) of these monkeys had positive titers to AN1792. Titers ranged from 2-246, with a mean of 49.44 ± 21.34. CSF anti-AN1792 levels were 0.18 ± 0.11% of what was measured in the serum and demonstrated a highly positive correlation (Spearman r = 0.7840) with serum titers. No differences were seen across groups or between sexes in the percentage of antibody in the CSF. The level of antibody in the CSF is consistent with the passive transfer of peripherally generated antibody across the blood-brain-barrier into the central nervous system.

Testing of a subset of anti-AN1792 positive CSF samples demonstrated that, like the antibody in serum samples, antibody in the CSF cross-reacts with Aβ1-40. Titers to Aβ1-40 showed a high correlation (Spearman r = 0.9634) to their respective AN1792 titers. Testing of a subset of CSF samples with the highest titers to AN1792 showed no binding to APP, as for the serum antibodies.

When sera from Day 175 was tested against a series of overlapping 10-mer peptides, antibodies from all of the monkeys bound to the peptide whose sequence covered

amino acids 1–10 of the AN1792 peptide (amino acids 653-672 of APP). In some animals, this was the only peptide to which binding could be measured (see Fig. 19).

In other animals, other reactivities could be measured, but in all cases the reactivity to the N-terminal peptide sequence was the predominant one. The additional reactivities fell into two groups. First and most common, was the binding to peptides centering around the N-terminal 1–10 AN1792 peptide (Figure 20). Binding of this type was directed to the peptides covering amino acids -1–8, -1–9, and 2–11 of the AN1792 peptide. These reactivities, combined with that to the 1–10 peptide, represent the overwhelming majority of reactivity in all animals. Epitope mapping of individual animals over time indicates that the antibody reactivity to the 1–10 peptide proceeds the spread to the adjacent peptides . This demonstrates a strong biasing of the immune response to the N-terminus of the AN1792 peptide with its free terminal aspartic acid residue. The second minor detectable activity in some animals was binding to peptides located C-terminally to the major area and centered around peptides covering amino acids 7–16, 11–20 and 16–25 of the AN1792 peptide . These reactivities were seen in only 10–30% of the monkeys.

Variability in response between different animals (e.g., whether amino acids 1-10 were the exclusive or predominant reactive epitope) did not correlate with antigen/adjuvant dose, dosing schedule, or antibody titer, and is probably a reflection of each individual animal's genetic make-up.

XVIII. PREVENTION AND TREATMENT OF HUMAN SUBJECTS

A single-dose phase I trial is performed to determine safety in humans. A therapeutic agent is administered in increasing dosages to different patients starting from about 0.01 the level of presumed efficacy, and increasing by a factor of three until a level of about 10 times the effective mouse dosage is reached.

A phase II trial is performed to determine therapeutic efficacy. Patients with early to mid Alzheimer's Disease defined using Alzheimer's disease and Related Disorders Association (ADRDA) criteria for probable AD are selected. Suitable patients score in the 12-26 range on the Mini-Mental State Exam (MMSE). Other selection criteria are that patients are likely to survive the duration of the study and lack complicating issues such as use of concomitant medications that may interfere. Baseline evaluations of patient function are made using classic psychometric measures, such as the MMSE, and the ADAS, which is a comprehensive scale for evaluating patients with Alzheimer's Disease status and function.

These psychometric scales provide a measure of progression of the Alzheimer's condition. Suitable qualitative life scales can also be used to monitor treatment. Disease progression can also be monitored by MRI. Blood profiles of patients can also be monitored including assays of immunogen-specific antibodies and T-cells responses.

Following baseline measures, patients begin receiving treatment. They are randomized and treated with either therapeutic agent or placebo in a blinded fashion. Patients are monitored at least every six months. Efficacy is determined by a significant reduction in progression of a treatment group relative to a placebo group.

A second phase II trial is performed to evaluate conversion of patients from non-Alzheimer's Disease early memory loss, sometimes referred to as age-associated memory impairment (AAMI) or mild cognitive impairment (MCI), to probable Alzheimer's disease as defined as by ADRDA criteria. Patients with high risk for conversion to Alzheimer's Disease are selected from a non-clinical population by screening reference populations for early signs of memory loss or other difficulties associated with pre-Alzheimer's symptomatology, a family history of Alzheimer's Disease, genetic risk factors, age, sex, and other features found to predict high-risk for Alzheimer's Disease. Baseline scores on suitable metrics including the MMSE and the ADAS together with other metrics designed to evaluate a more normal population are collected. These patient populations are divided into suitable groups with placebo comparison against dosing alternatives with the agent. These patient populations are followed at intervals of about six months, and the endpoint for each patient is whether or not he or she converts to probable Alzheimer's Disease as defined by ADRDA criteria at the end of the observation.

XIX. General Materials and Methods

1. Measurement of Antibody Titers

Mice were bled by making a small nick in the tail vein and collecting about 200 µl of blood into a microfuge tube. Guinea pigs were bled by first shaving the back hock area and then using an 18 gauge needle to nick the metatarsal vein and collecting the blood into microfuge tubes. Blood was allowed to clot for one hr at room temperature (RT), vortexed, then centrifuged at 14,000 x g for 10 min to separate the clot from the serum. Serum was then transferred to a clean microfuge tube and stored at 4°C until titered.

Antibody titers were measured by ELISA. 96-well microtiter plates (Costar EIA plates) were coated with 100 µl of a solution containing either 10 µg/ml either Aβ42 or

SAPP or other antigens as noted in each of the individual reports in Well Coating Buffer (0.1 M sodium phosphate, pH 8.5, 0.1% sodium azide) and held overnight at RT. The wells were aspirated and sera were added to the wells starting at a 1/100 dilution in Specimen Diluent (0.014 M sodium phosphate, pH 7.4, 0.15 M NaCl, 0.6% bovine serum albumin, 0.05% thimerosal). Seven serial dilutions of the samples were made directly in the plates in three-fold steps to reach a final dilution of 1/218,700. The dilutions were incubated in the coated-plate wells for one hr at RT. The plates were then washed four times with PBS containing 0.05% Tween 20. The second antibody, a goat anti-mouse Ig conjugated to horseradish peroxidase (obtained from Boehringer Mannheim), was added to the wells as 100 µl of a 1/3000 dilution in Specimen Diluent and incubated for one hr at RT. Plates were again washed four times in PBS, Tween 20. To develop the chromogen, 100 µl of Slow TMB (3,3',5,5'-tetramethyl benzidine obtained from Pierce Chemicals) was added to each well and incubated for 15 min at RT. The reaction was stopped by the addition of 25 µl of 2 M H_2SO_4. The color intensity was then read on a Molecular Devices Vmax at (450 nm - 650 nm).

Titers were defined as the reciprocal of the dilution of serum giving one half the maximum OD. Maximal OD was generally taken from an initial 1/100 dilution, except in cases with very high titers, in which case a higher initial dilution was necessary to establish the maximal OD. If the 50% point fell between two dilutions, a linear extrapolation was made to calculate the final titer. To calculate geometric mean antibody titers, titers less than 100 were arbitrarily assigned a titer value of 25.

2. Lymphocyte proliferation assay

Mice were anesthetized with isoflurane. Spleens were removed and rinsed twice with 5 ml PBS containing 10% heat-inactivated fetal bovine serum (PBS-FBS) and then homogenized in a 50° Centricon unit (Dako A/S, Denmark) in 1.5 ml PBS-FBS for 10 sec at 100 rpm in a Medimachine (Dako) followed by filtration through a 100 micron pore size nylon mesh. Splenocytes were washed once with 15 ml PBS-FBS, then pelleted by centrifugation at 200 x g for 5 min. Red blood cells were lysed by resuspending the pellet in 5 mL buffer containing 0.15 M NH4Cl, 1 M KHCO3, 0.1 M NaEDTA, pH 7.4 for five min at RT. Leukocytes were then washed as above. Freshly isolated spleen cells (10^5 cells per well) were cultured in triplicate sets in 96-well U-bottomed tissue culture-treated microtiter plates (Corning, Cambridge, MA) in RPMI 1640 medium (JRH Biosciences, Lenexa, KS)

supplemented with 2.05 mM L glutamine, 1% Penicillin/Streptomycin, and 10% heat-inactivated FBS, for 96 hr at 37°C. Various Aβ peptides, Aβ1-16, Aβ1-40, Aβ1-42 or Aβ40-1 reverse sequence protein were also added at doses ranging from 5 to 0.18 micromolar in four steps. Cells in control wells were cultured with Concanavalin A (Con A) (Sigma, cat. # C-5275, at 1 microgram/ml) without added protein. Cells were pulsed for the final 24 hr with 3H-thymidine (1 μCi/well obtained from Amersham Corp., Arlington Heights IL). Cells were then harvested onto UniFilter plates and counted in a Top Count Microplate Scintillation Counter (Packard Instruments, Downers Grove, IL). Results are expressed as counts per minute (cpm) of radioactivity incorporated into insoluble macromolecules.

4. Brain Tissue Preparation

After euthanasia, the brains were removed and one hemisphere was prepared for immunohistochemical analysis, while three brain regions (hippocampus, cortex and cerebellum) were dissected from the other hemisphere and used to measure the concentration of various Aβ proteins and APP forms using specific ELISAs (Johnson-Wood et al., supra).

Tissues destined for ELISAs were homogenized in 10 volumes of ice-cold guanidine buffer (5.0 M guanidine-HCl, 50 mM Tris-HCl, pH 8.0). The homogenates were mixed by gentle agitation using an Adams Nutator (Fisher) for three to four hr at RT, then stored at -20°C prior to quantitation of Aβ and APP. Previous experiments had shown that the analytes were stable under this storage condition, and that synthetic Aβ protein (Bachem) could be quantitatively recovered when spiked into homogenates of control brain tissue from mouse littermates (Johnson-Wood et al., supra).

5. Measurement of Aβ Levels

The brain homogenates were diluted 1:10 with ice cold Casein Diluent (0.25% casein, PBS, 0.05% sodium azide, 20 μg/ml aprotinin, 5 mM EDTA pH 8.0, 10 μg/ml leupeptin) and then centrifuged at 16,000 x g for 20 min at 4° C. The synthetic Aβ protein standards (1-42 amino acids) and the APP standards were prepared to include 0.5 M guanidine and 0.1% bovine serum albumin (BSA) in the final composition. The "total" Aβ sandwich ELISA utilizes monoclonal antibody monoclonal antibody 266, specific for amino acids 13-28 of Aβ (Seubert, et al.), as the capture antibody, and biotinylated monoclonal antibody 3D6, specific for amino acids 1-5 of Aβ (Johnson-Wood, et al), as the reporter

antibody. The 3D6 monoclonal antibody does not recognize secreted APP or full-length APP, but detects only Aβ species with an amino-terminal aspartic acid. This assay has a lower limit of sensitivity of ~50 ng/ml (11nM) and shows no cross-reactivity to the endogenous murine Aβ protein at concentrations up to 1 ng/ml (Johnson-Wood et al., supra).

The Aβ1-42 specific sandwich ELISA employs mAβ 21F12, specific for amino acids 33-42 of Aβ (Johnson-Wood, et al.), as the capture antibody. Biotinylated mAβ 3D6 is also the reporter antibody in this assay which has a lower limit of sensitivity of about 125 μg/ml (28 μM, Johnson-Wood et al.). For the Aβ ELISAs, 100 μl of either mAβ 266 (at 10 μg/ml) or mAβ 21F12 at (5 μg/ml) was coated into the wells of 96-well immunoassay plates (Costar) by overnight incubation at RT. The solution was removed by aspiration and the wells were blocked by the addition of 200 μl of 0.25% human serum albumin in PBS buffer for at least 1 hr at RT. Blocking solution was removed and the plates were stored desiccated at 4°C until used. The plates were rehydrated with Wash Buffer [Tris-buffered saline (0.15 M NaCl, 0.01 M Tris-HCl, pH 7.5), plus 0.05% Tween 20] prior to use. The samples and standards were added in triplicate aliquots of 100 μl per well and then incubated overnight at 4° C. The plates were washed at least three times with Wash Buffer between each step of the assay. The biotinylated mAβ 3D6, diluted to 0.5 μg/ml in Casein Assay Buffer (0.25% casein, PBS, 0.05% Tween 20, pH 7.4), was added and incubated in the wells for 1 hr at RT. An avidin-horseradish peroxidase conjugate, (Avidin-HRP obtained from Vector, Burlingame, CA), diluted 1:4000 in Casein Assay Buffer, was added to the wells for 1 hr at RT. The colorimetric substrate, Slow TMB-ELISA (Pierce), was added and allowed to react for 15 minutes at RT, after which the enzymatic reaction was stopped by the addition of 25 μl 2 N H2SO4. The reaction product was quantified using a Molecular Devices Vmax measuring the difference in absorbance at 450 nm and 650 nm.

6. Measurement of APP Levels

Two different APP assays were utilized. The first, designated APP-α/FL, recognizes both APP-alpha (α) and full-length (FL) forms of APP. The second assay is specific for APP-α. The APP-α /FL assay recognizes secreted APP including the first 12 amino acids of Aβ. Since the reporter antibody (2H3) is not specific to the α-clip-site, occurring between amino acids 612-613 of APP695 (Esch et al., Science 248, 1122-1124 (1990)); this assay also recognizes full length APP (APP-FL). Preliminary experiments using

immobilized APP antibodies to the cytoplasmic tail of APP-FL to deplete brain homogenates of APP-FL suggest that approximately 30-40% of the APP-α /FL APP is FL (data not shown). The capture antibody for both the APP-α/FL and APP-α assays is mAb 8E5, raised against amino acids 444 to 592 of the APP695 form (Games et al., supra). The reporter mAb for the APP-α/FL assay is mAb 2H3, specific for amino acids 597-608 of APP695 (Johnson-Wood et al., supra) and the reporter antibody for the APP-α assay is a biotinylated derivative of mAb 16H9, raised to amino acids 605 to 611 of APP. The lower limit of sensitivity of the APP-αFL assay is about 11 ng/ml (150 ρM) (Johnson-Wood et al.) and that of the APP-α specific assay is 22 ng/ml (0.3 nM). For both APP assays, mAb 8E5 was coated onto the wells of 96-well EIA plates as described above for mAb 266. Purified, recombinant secreted APP-α was used as the reference standard for the APP-α assay and the APP-α/FL assay (Esch et al., supra). The brain homogenate samples in 5 M guanidine were diluted 1:10 in ELISA Specimen Diluent (0.014 M phosphate buffer, pH 7.4, 0.6% bovine serum albumin, 0.05% thimerosal, 0.5 M NaCl, 0.1% NP40). They were then diluted 1:4 in Specimen Diluent containing 0.5 M guanidine. Diluted homogenates were then centrifuged at 16,000 x g for 15 seconds at RT. The APP standards and samples were added to the plate in duplicate aliquots and incubated for 1.5 hr at RT. The biotinylated reporter antibody 2H3 or 16H9 was incubated with samples for 1 hr at RT. Streptavidin-alkaline phosphatase (Boehringer Mannheim), diluted 1:1000 in specimen diluent, was incubated in the wells for 1 hr at RT. The fluorescent substrate 4-methyl-umbellipheryl-phosphate was added for a 30-min RT incubation and the plates were read on a Cytofluor tm 2350 fluorimeter (Millipore) at 365 nm excitation and 450 nm emission.

7. Immunohistochemistry

Brains were fixed for three days at 40C in 4% paraformaldehyde in PBS and then stored from one to seven days at 4°C in 1% paraformaldehyde, PBS until sectioned. Forty-micron-thick coronal sections were cut on a vibratome at RT and stored in cryoprotectant (30% glycerol, 30% ethylene glycol in phosphate buffer) at -20°C prior to immunohistochemical processing. For each brain, six sections at the level of the dorsal hippocampus, each separated by consecutive 240 μm intervals, were incubated overnight with one of the following antibodies: (1) a biotinylated anti-Aβ (mAb, 3D6, specific for human Aβ) diluted to a concentration of 2 μg/ml in PBS and 1% horse serum; or (2) a biotinylated mAb specific for human APP, 8E5, diluted to a concentration of 3 μg/ml in PBS

and 1.0% horse serum; or (3) a mAb specific for glial fibrillary acidic protein (GFAP; Sigma Chemical Co.) diluted 1:500 with 0.25% Triton X-100 and 1% horse serum, in Tris-buffered saline, pH 7.4 (TBS); or (4) a mAb specific for CD11b, MAC-1 antigen, (Chemicon International) diluted 1:100 with 0.25% Triton X-100 and 1% rabbit serum in TBS; or (5) a mAb specific for MHC II antigen, (Pharmingen) diluted 1:100 with 0.25% Triton X-100 and 1% rabbit serum in TBS; or (6) a rat mAb specific for CD 43 (Pharmingen) diluted 1:100 with 1% rabbit serum in PBS or (7) a rat mAb specific for CD 45RA (Pharmingen) diluted 1:100 with 1% rabbit serum in PBS; or (8) a rat monoclonal Aβ specific for CD 45RB (Pharmingen) diluted 1:100 with 1% rabbit serum in PBS; or (9) a rat monoclonal Aβ specific for CD 45 (Pharmingen) diluted 1:100 with 1% rabbit serum in PBS; or (10) a biotinylated polyclonal hamster Aβ specific for CD3e (Pharmingen) diluted 1:100 with 1% rabbit serum in PBS or (11) a rat mAb specific for CD3 (Serotec) diluted 1:200 with 1% rabbit serum in PBS; or with (12) a solution of PBS lacking a primary antibody containing 1% normal horse serum.

Sections reacted with antibody solutions listed in 1,2 and 6-12 above were pretreated with 1.0% Triton X-100, 0.4% hydrogen peroxide in PBS for 20 min at RT to block endogenous peroxidase. They were next incubated overnight at 4°C with primary antibody. Sections reacted with 3D6 or 8E5 or CD3e mAbs were then reacted for one hr at RT with a horseradish peroxidase-avidin-biotin-complex with kit components "A" and "B" diluted 1:75 in PBS (Vector Elite Standard Kit, Vector Labs, Burlingame, CA.). Sections reacted with antibodies specific for CD 45RA, CD 45RB, CD 45, CD3 and the PBS solution devoid of primary antibody were incubated for 1 hour at RT with biotinylated anti-rat IgG (Vector) diluted 1:75 in PBS or biotinylated anti-mouse IgG (Vector) diluted 1:75 in PBS, respectively. Sections were then reacted for one hr at RT with a horseradish peroxidase-avidin-biotin-complex with kit components "A" and "B" diluted 1:75 in PBS (Vector Elite Standard Kit, Vector Labs, Burlingame, CA.).

Sections were developed in 0.01% hydrogen peroxide, 0.05% 3,3'-diaminobenzidine (DAB) at RT. Sections destined for incubation with the GFAP-, MAC-1- AND MHC II-specific antibodies were pretreated with 0.6% hydrogen peroxide at RT to block endogenous peroxidase then incubated overnight with the primary antibody at 4°C. Sections reacted with the GFAP antibody were incubated for 1 hr at RT with biotinylated anti-mouse IgG made in horse (Vector Laboratories; Vectastain Elite ABC Kit) diluted 1:200 with TBS. The sections were next reacted for one hr with an avidin-biotin-peroxidase

complex (Vector Laboratories; Vectastain Elite ABC Kit) diluted 1:1000 with TBS. Sections incubated with the MAC-1-or MHC II-specific monoclonal antibody as the primary antibody were subsequently reacted for 1 hr at RT with biotinylated anti-rat IgG made in rabbit diluted 1:200 with TBS, followed by incubation for one hr with avidin-biotin-peroxidase complex diluted 1:1000 with TBS. Sections incubated with GFAP-, MAC-1- and MHC II-specific antibodies were then visualized by treatment at RT with 0.05% DAB, 0.01% hydrogen peroxide, 0.04% nickel chloride, TBS for 4 and 11 min, respectively.

Immunolabeled sections were mounted on glass slides (VWR, Superfrost slides), air dried overnight, dipped in Propar (Anatech) and overlaid with coverslips using Permount (Fisher) as the mounting medium.

To counterstain Aβ plaques, a subset of the GFAP-positive sections were mounted on Superfrost slides and incubated in aqueous 1% Thioflavin S (Sigma) for 7 min following immunohistochemical processing. Sections were then dehydrated and cleared in Propar, then overlaid with coverslips mounted with Permount.

8. Image Analysis

A Videometric 150 Image Analysis System (Oncor, Inc., Gaithersburg, MD) linked to a Nikon Microphot-FX microscope through a CCD video camera and a Sony Trinitron monitor was used for quantification of the immunoreactive slides. The image of the section was stored in a video buffer and a color-and saturation-based threshold was determined to select and calculate the total pixel area occupied by the immunolabeled structures. For each section, the hippocampus was manually outlined and the total pixel area occupied by the hippocampus was calculated. The percent amyloid burden was measured as: (the fraction of the hippocampal area containing Aβ deposits immunoreactive with mAb 3D6) x 100. Similarly, the percent neuritic burden was measured as: (the fraction of the hippocampal area containing dystrophic neurites reactive with monoclonal antibody 8E5) x100. The C-Imaging System (Compix, Inc., Cranberry Township, PA) operating the Simple 32 Software Application program was linked to a Nikon Microphot-FX microscope through an Optronics camera and used to quantitate the percentage of the retrospenial cortex occupied by GFAP-positive astrocytes and MAC-1-and MHC II-positive microglia. The image of the immunoreacted section was stored in a video buffer and a monochrome-based threshold was determined to select and calculate the total pixel area occupied by immunolabeled cells. For each section, the retrosplenial cortex (RSC) was manually outlined and the total pixel area

occupied by the RSC was calculated. The percent astrocytosis was defined as: (the fraction of RSC occupied by GFAP-reactive astrocytes) X 100. Similarly, percent microgliosis was defined as: (the fraction of the RSC occupied by MAC-1- or MHC II-reactive microglia) X 100. For all image analyses, six sections at the level of the dorsal hippocampus, each separated by consecutive 240 μm intervals, were quantitated for each animal. In all cases, the treatment status of the animals was unknown to the observer.

Although the foregoing invention has been described in detail for purposes of clarity of understanding, it will be obvious that certain modifications may be practiced within the scope of the appended claims. All publications and patent documents cited herein are hereby incorporated by reference in their entirety for all purposes to the same extent as if each were so individually denoted.

From the foregoing it will be apparent that the invention provides for a number of uses. For example, the invention provides for the use of any of the antibodies to Aβ described above in the treatment, prophylaxis or diagnosis of amyloidogenic disease, or in the manufacture of a medicament or diagnostic composition for use in the same. Likewise, the invention provides for the use of any of the epitopic fragments of Aβ described above for the treatment or prophylaxis of amyloidogenic disease or in the manufacture of a medicament for use in the same.

TABLE 1

TITER AT 50% MAXIMAL O.D.

Aggreated Aß injected mice

Age of PDAPP	mouse 100	mouse 101	mouse 102	mouse 103	mouse 104	mouse 105	mouse 106	mouse 107	mouse 108
4	70000	150000	15000	120000	1000	15000	50000	80000	100000
6	15000	65000	30000	55000	300	15000	15000	50000	60000
8	20000	55000	50000	50000	400	15000	18000	50000	60000
10	40000	20000	60000	50000	900	15000	50000	20000	40000
12	25000	30000	60000	40000	2700	20000	70000	25000	20000

PBS injected mice on both immunogens at 1/100

Age of PDAPP	mouse 113	mouse 114	mouse115	mouse116	mouse117
6	< 4x bkg	< 4x bkg	< 4x bkg	< 4x bkg	< 4x bkg
10	5 x bkg	< 4x bkg	< 4x bkg	< 4x bkg	< 4x bkg
12	< 4x bkg	< 4x bkg	< 4x bkg	< 4x bkg	< 4x bkg

WHAT IS CLAIMED IS:

1. A method of preventing or treating a disease associated with amyloid deposits of aβ in the brain of a patient, comprising administering an effective dosage of an antibody that binds to aβ to the patient.

2. The method of claim 1, wherein the disease is characterized by cognitive impairment.

3. The method of claim 1, wherein the disease is Alzheimer's disease.

4. The method of claim 1, wherein the disease is Down's syndrome.

5. The method of claim 1, wherein the disease is mild cognitive impairment.

6. The method of claim 1, wherein the antibody is of human isotype IgG1.

7. The method of any of the preceding claims, wherein the patient is human.

8. The method of any of the preceding claims, wherein the antibody specifically binds to an epitope within residues 1-10 of Aβ.

9. The method of any of claims 1-8 , wherein the antibody specifically binds to an epitope within residues 1-6 of Aβ.

10. The method of any of claims 1-8, wherein the antibody specifically binds to an epitope within residues 1-5 of Aβ.

11. The method of any of claims 1-8, wherein the antibody specifically binds to an epitope within residues 1-7 of Aβ.

12. The method of any of claims 1-8, wherein the antibody specifically binds to an epitope within residues 3-7 of Aβ.

13. The method of any of claims 1-8, wherein the antibody specifically binds to an epitope within residues 1-3 of Aβ.

14. The method of any of claims 1-8, wherein the antibody specifically binds to an epitope within residues 1-4 of Aβ.

15. The method of any of the preceding claims, wherein after administration the antibody binds to an amyloid deposit in the patient and induces a clearing response against the amyloid deposit.

16. The method of claim 15, wherein the clearing response is an Fc receptor mediated phagocytosis response.

17. The method of claim 15 or 16, further comprising monitoring the clearing response.

18. The method of any of the preceding claims, wherein the antibody specifically binds to an epitope comprising a free N-terminal residue of Aβ.

19. The method of any of the preceding claims, where in the antibody binds to an epitope within residues of 1-10 of Aß wherein residue 1 and/or residue 7 of Aβ is iso-aspartic acid.

20. The method of any of the preceding claims, wherein the patient is asymptomatic.

21. The method of any of the preceding claims, wherein the patient is under 50.

22. The method of any of the preceding claims, wherein the patient has an inherited risk factor indicating susceptibility to Alzheimer's disease.

23. The method of any of claims 1-22, wherein the patient has no known risk factors for Alzheimer's disease.

24. The method of any of the preceding claims, wherein the antibody is a human antibody.

25. The method of any of claims 1-23, wherein the antibody is a humanized antibody.

26. The method of any of claims 1-23, wherein the antibody is a chimeric antibody.

27. The method of any of claims 1-23, wherein the antibody is a mouse antibody.

28. The method of any of the preceding claims, wherein the antibody is a polyclonal antibody.

29. The method of any of claims 1-27, wherein the antibody is a monoclonal antibody.

30. The method of any of the preceding claims, further comprising administering an effective dosage of at least one other antibody that binds to a different epitope of Aβ.

31. The method of claims 1-5 or claims 7-30, wherein the isotype of the antibody is IgG1 or IgG4.

32. The method of any of claims 1-5 or 7-30, wherein the isotype of the antibody is IgG2 or IgG3.

33. The method of any of claims 1-32, wherein the antibody comprises two copies of the same pair of light and heavy chains.

34. The method of any of claims 1-32, wherein the antibody is a bispecific antibody comprising a first light and heavy chain pair that specifically binds to the epitope of Aβ and a second light and heavy chain pair that specifically binds to an Fc receptor on microglial cells.

35. The method of any of the preceding claims, wherein a chain of the antibody is fused to a heterologous polypeptide.

36. The method of any of the preceding claims, wherein the dosage of antibody is at least 1 mg/kg body weight of the patient.

37. The method of claim 36, wherein the dosage of antibody is at least 10 mg/kg body weight of the patient.

38. The method of any of the preceding claims, wherein the antibody is administered with a carrier as a pharmaceutical composition.

39. The method of claims 1-24, 28 and 29-38, wherein the antibody is a human antibody to Aβ prepared from B cells from a human immunized with an Aβ peptide.

40. The method of claim 39, wherein the human immunized with Aβ peptide is the patient.

41. The method of any of the preceding claims, wherein the antibody specifically binds to Aβ peptide without binding to full-length amyloid precursor protein (APP).

42. The method of any of the preceding claims, wherein the antibody is administered intraperitoneally, orally, intranasally, subcutaneously, intramuscularly, topically or intravenously.

43. The method of any of the preceding claims, wherein the antibody is administered by administering a polynucleotide encoding at least one antibody chain to the patient, wherein the polynucleotide is expressed to produce the antibody chain in the patient.

44. The method of claim 43, wherein the polynucleotide encodes heavy and light chains of the antibody, which polynucleotide is expressed to produce the heavy and light chains in the patient.

45. The method of any of the preceding claims, further comprising monitoring the patient for level of administered antibody in the blood of the patient.

46. The method of any of the preceding claims, wherein the antibody is administered in multiple dosages over a period of at least six months.

47. The method of any of claims 1-45, wherein the antibody is administered as a sustained release composition.

48. A method of preventing or treating a disease associated with amyloid deposits of Aβ in the brain of a patient, comprising administering to the patient an effective dosage of a polypeptide comprising an N-terminal segment of at least residues 1-5 of Aβ, the first residue of Aβ being the N-terminal residue of the polypeptide, wherein the polypeptide is free of a C-terminal segment of Aβ.

49. The method of claim 48, wherein the disease is characterized by cognitive impairment.

50. The method of claim 48, wherein the disease is Alzheimer's disease.

51. The method of claim 48, wherein the disease is Down's syndrome.

52. The method of claim 48, wherein the disease is mild cognitive impairment.

53. A method of preventing or treating associated with amyloid deposits of Aβ in the brain of a patient, comprising administering to the patient an effective dosage of a polypeptide comprising an N-terminal segment of Aβ, the segment beginning at residue 1-3 of Aβ and ending at residues 7-11 of Aβ.

54. The method of claim 53, wherein the disease is characterized by cognitive impairment.

55. The method of claim 53, wherein the disease is Alzheimer's disease.

56. The method of claim 53, wherein the disease is Down's syndrome.

57. The method of claim 53, wherein the disease is mild cognitive impairment.

58 The method of any of claims 48-57, wherein the N-terminal segment of Aβ is linked at its C-terminus to a heterologous polypeptide.

59. The method of claim 58, wherein the N-terminal segment consists of the amino acid sequence DAEFRHD.

60. The method of claim 59, wherein the polypeptide comprises the amino acid sequence DAEFRHDQYIKANSKFIGITEL.

61. The method of claim 53, wherein the N-terminal segment of Aβ is linked at its N-terminus to a heterologous polypeptide.

62. The method of claim 61, wherein the polypeptide comprises the amino acid sequence AKXVAAWTLKAAADAEFRHD.

63. The method of claim 53, wherein the N-terminal segment of Aβ is linked at its N and C termini to first and second heterologous polypeptides.

64. The method claim 53, wherein the N-terminal segment of Aβ is linked at its N terminus to a heterologous polypeptide, and at its C-terminus to at least one additional copy of the N-terminal segment.

65. The method of any of claims 58-62 and 64, wherein the heterologous polypeptide induces a T-cell response against the heterologous polypeptide and thereby a B-cell response against the N-terminal segment.

66. The method of any of claims 48-62 and 65, wherein the polypeptide further comprises at least one additional copy of the N-terminal segment.

67. The method of claim 53, wherein the polypeptide comprises from N-terminus to C-terminus, the N-terminal segment of Aβ, a plurality of additional copies of the N-terminal segment, and the heterologous amino acid segment.

68. The method of any of claims 48-67, wherein the N-terminal segment consists of Aβ1-7.

69. The method of any of claims 48-58, 61, and 63, wherein the N-terminal segment consists of Aβ3-7.

70. The method of any of claims 48-57, wherein the polypeptide consists of Aβ1-7.

71. The method of any of claims 48-57, wherein the polypeptide consists of Aβ3-7.

71. The method of any of claims 48-67, wherein the polypeptide is free of at least the 5 C-terminal amino acids in Aβ43.

72. The method of any of claims 48-71, wherein the polypeptide is administered with an adjuvant that enhances an immune response to the N-terminal segment.

73. The method of claim 72, wherein the adjuvant and the polypeptide are administered together as a composition.

74. The method of claim 72, wherein the adjuvant is administered before the polypeptide.

75. The method of claim 72, wherein the adjuvant is administered after the polypeptide.

76. The method of any of claims 72-75, wherein the adjuvant is alum.

77. The method of any of claims 72-75, wherein the adjuvant is MPL.

78. The method of any of claims 72-75, wherein the adjuvant is QS-21.

79. The method of any of claims 72-75, wherein the adjuvant is incomplete Freund's adjuvant.

80. The method of any of claims 48-79, wherein the dosage of the polypeptide is greater than 10 micrograms.

81. A method of preventing or treating a disease associated with amyloid deposits of Aβ in the brain of a patient, comprising administering to a patient an effective dosage of an agent that induces an immunogenic response against an N-terminal segment of Aβ, the segment beginning at residue 1-3 of Aβ and ending at residues 7-11 of Aβ without inducing an immunogenic response against an epitope within residues 12-43 of Aβ43.

82. The method of claim 81, wherein the disease is characterized by cognitive impairment.

83. The method of claim 81, wherein the disease is Alzheimer's disease.

84. The method of claim 81, wherein the disease is Down's syndrome.

85. The method of claim 81, wherein the disease is mild cognitive impairment.

86. A pharmaceutical composition comprising a polypeptide as defined in any of claims 48-71 and an adjuvant.

87. A pharmaceutical composition comprising an antibody as defined in any of claims 1-19, 24-29, 31-35, 39 and 40, and a pharmaceutically acceptable carrier.

88. A method of screening an antibody for activity in treating a disease associated with amyloid deposits of Aβ in the brain of a patient, comprising

contacting the antibody with a polypeptide comprising at least five contiguous amino acids of an N-terminal segment of Aβ beginning at a residue between 1 and 3 of Aβ, the polypeptide being free of a C-terminal segment of Aβ,

and determining whether the antibody specifically binds to the polypeptide, specific binding providing an indication that the antibody has activity in treating Alzheimer's disease.

89. The method of claim 88, wherein the disease is Alzheimer's disease.

90. A method of screening an antibody for activity in clearing a biological entity physically associated with an antigen, comprising

combining the antigen-associated biological entity, the antibody and phagocytic cells bearing Fc receptors in a medium;

monitoring the amount of the antigen-associated biological entity remaining in the medium, a reduction in amount of the antigen-associated biological entity indicating the antibody has clearing activity against the antigen.

91. The method of claim 90, wherein the monitoring step monitors the amount of the antigen remaining in the medium.

92. The method of claim 90 or 91, wherein the combining comprises adding antigen-associated biological entity to the medium, and contacting the medium with the phagocytic cells bearing Fc receptors.

93. The method of any of claims 90-92, wherein the antigen-associated biological entity is provided as a tissue sample.

94. The method of any of claims 90-93, wherein the antigen is the biological entity.

95. The method of claim 94, wherein the tissue sample comprises an amyloid deposit.

96. The method of any of claims 93-95, wherein the tissue sample is from the brain of an Alzheimer's disease patient or a mammal having Alzheimer's pathology.

97. The method of any of claims 90-96, wherein the antigen is Aβ.

98. The method of any of claims 90-97, wherein the phagocytic cells are microglial cells.

99. The method of any of claims 93-95, wherein the tissue sample is selected from the group consisting of a cancerous tissue sample, a virally infected tissue sample, a tissue sample comprising inflammatory cells, a nonmalignant abnormal cell growth, and a tissue sample comprising an abnormal extracellular matrix.

100. A method of detecting an amyloid deposit in a patient, comprising

administering to the patient an antibody that specifically binds to an epitope within amino acids 1-10 of Aβ;

detecting presence of the antibody in the brain of the patient.

101. The method of claim 100, wherein the antibody binds to an epitope within residues 4-10 of Aβ.

102. The method of claim 100, wherein the antibody binds to an epitope within residues 8-10 of Aβ.

103. The method of any of claims 100-102, wherein the antibody is labelled.

104. The method of claim 103, wherein the antibody is labelled with a paramagnetic label.

105. The method of claim 104, wherein the labelled antibody is detected by nuclear magnetic resonance.

106. The method of any of claims 100-105, wherein the antibody lacks capacity to induce a clearance response on binding to an amyloid deposit in the patient.

107. A diagnostic kit, comprising
an antibody that specifically binds to an epitope with residues 1-10 of Aβ.

108. The kit of claim 107, further comprising labeling describing use of the antibody for in vivo diagnosis or monitoring of a disease associated with amyloid deposits of Aβ in the brain of a patient.

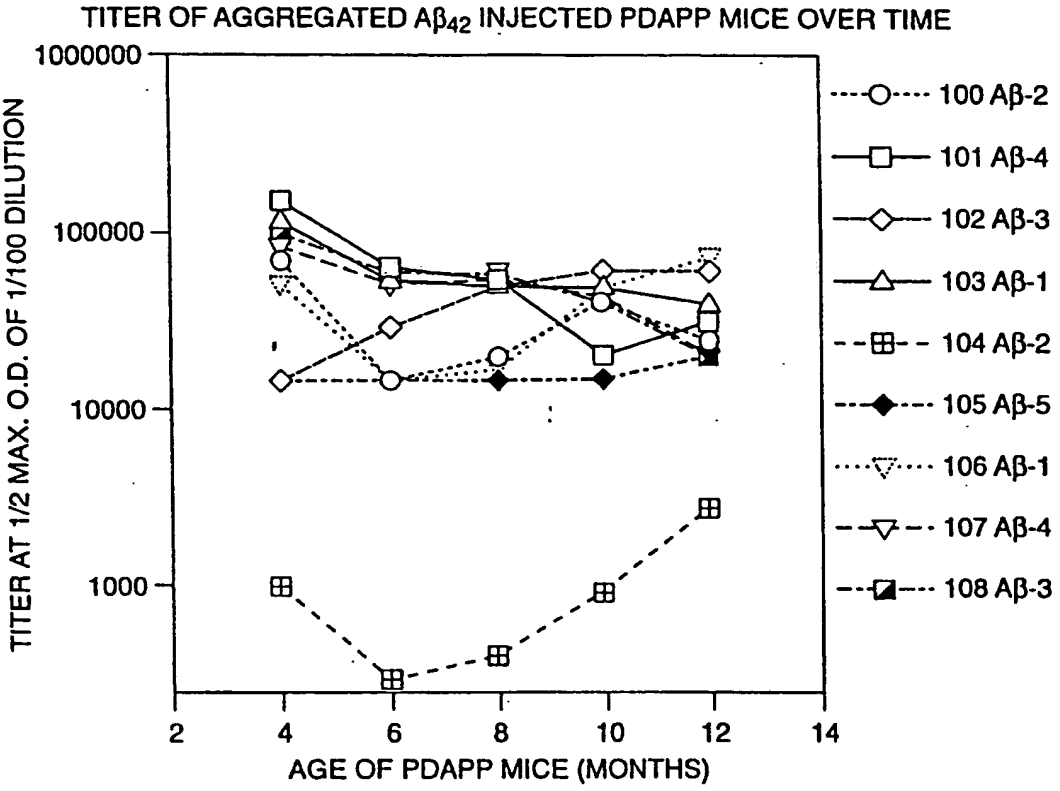

TITER OF AGGREGATED Aβ42 INJECTED PDAPP MICE OVER TIME
TITER AT 1/2 MAX. O.D. OF 1/100 DILUTION
1000000
100000
10000
1000
2
4
6
8
10
12
14
AGE OF PDAPP MICE (MONTHS)
100 Aβ-2
101 Aβ-4
102 Aβ-3
103 Aβ-1
104 Aβ-2
105 Aβ-5
106 Aβ-1
107 Aβ-4
108 Aβ-3

FIG. 1

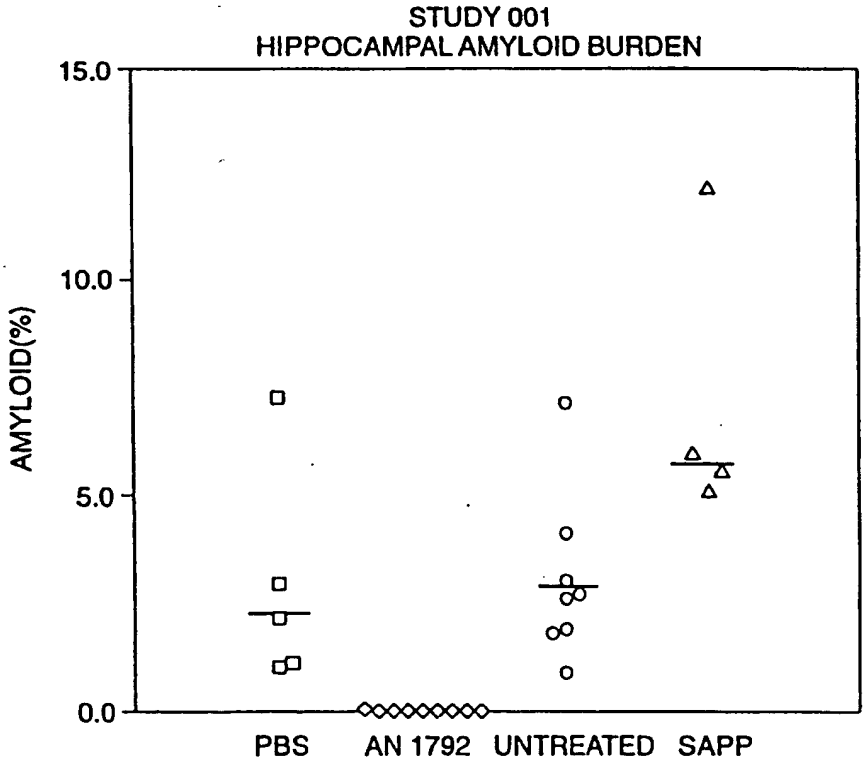

STUDY 001
HIPPOCAMPAL AMYLOID BURDEN
AMYLOID(%)
15.0
10.0
5.0
0.0
PBS
AN 1792
UNTREATED SAPP

FIG. 2

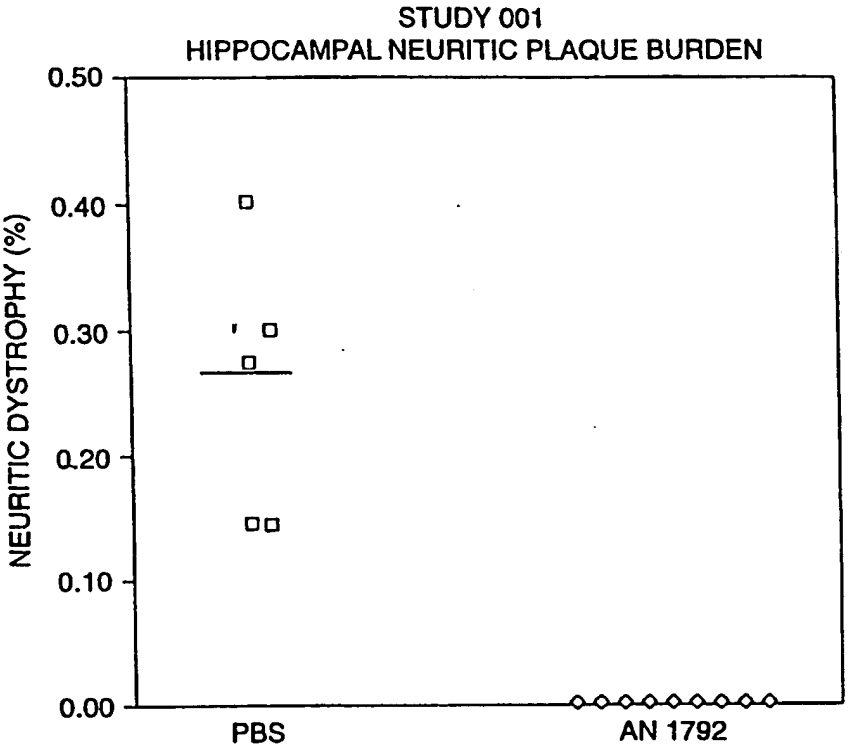

STUDY 001
HIPPOCAMPAL NEURITIC PLAQUE BURDEN
NEURITIC DYSTROPHY (%)
0.50
0.40
0.30
0.20
0.10
0.00
PBS
AN 1792

FIG. 3

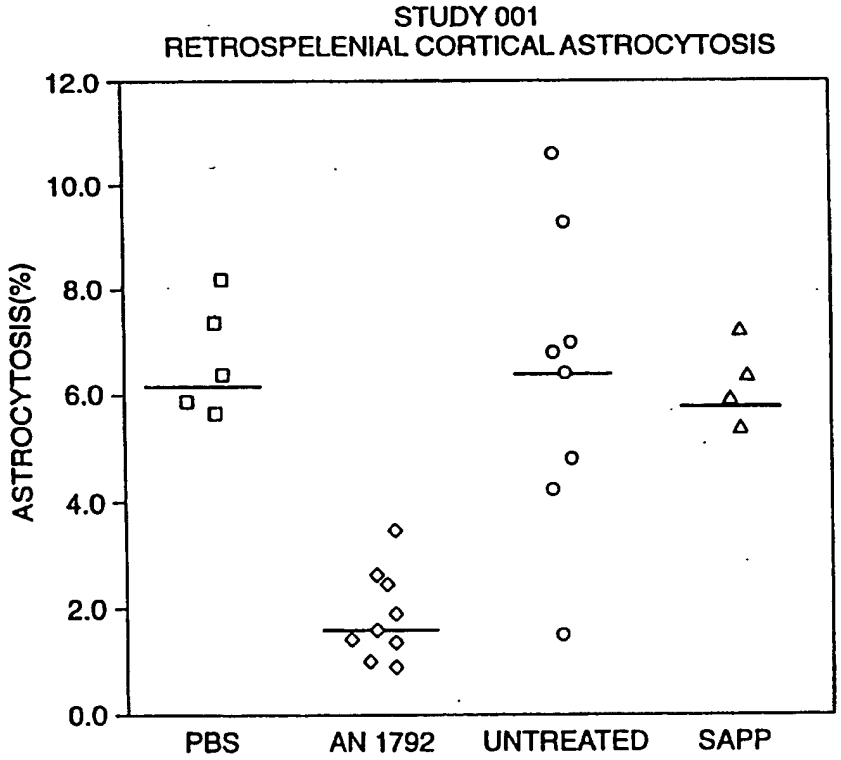

STUDY 001
RETROSPELENIAL CORTICAL ASTROCYTOSIS
ASTROCYTOSIS(%)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
PBS
AN 1792
UNTREATED
SAPP

FIG. 4

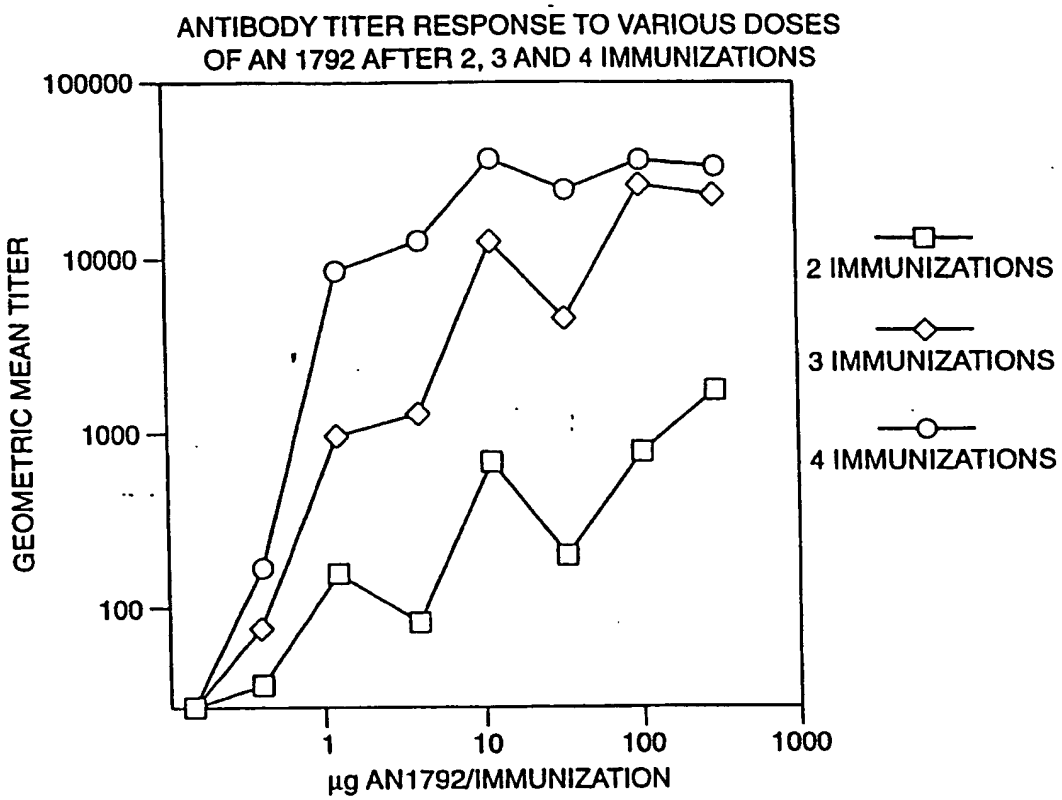

ANTIBODY TITER RESPONSE TO VARIOUS DOSES
OF AN 1792 AFTER 2, 3 AND 4 IMMUNIZATIONS
GEOMETRIC MEAN TITER
100000
10000
1000
100
1
10
100
1000
µg AN1792/IMMUNIZATION
2 IMMUNIZATIONS
3 IMMUNIZATIONS
4 IMMUNIZATIONS

FIG. 5

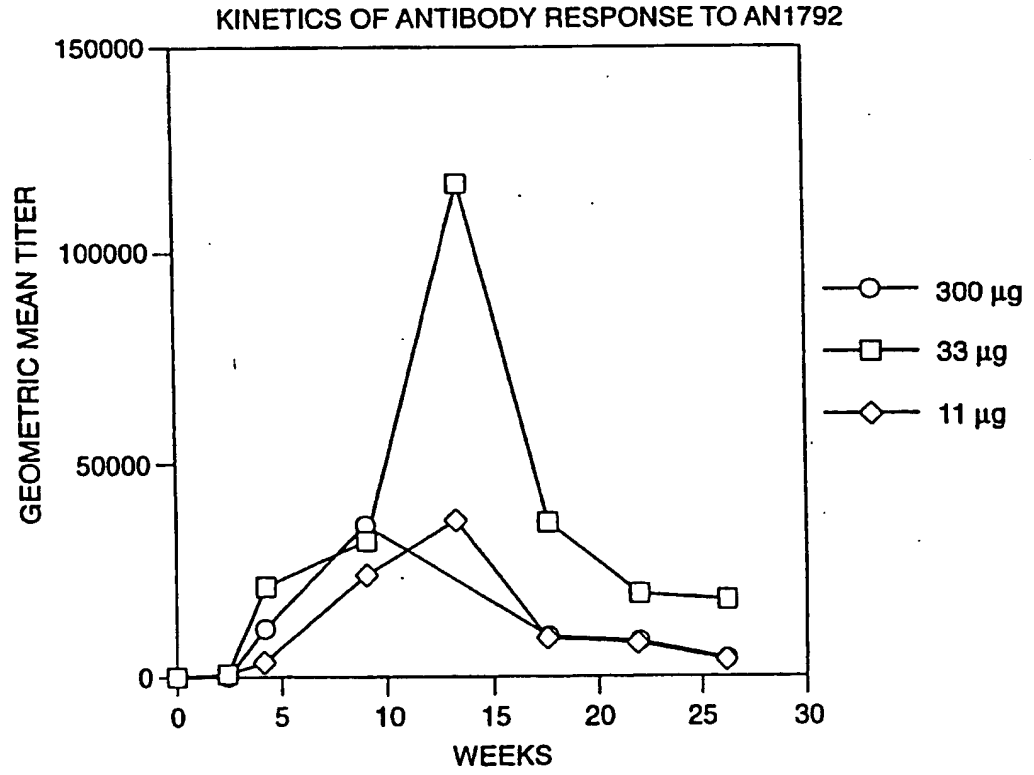

KINETICS OF ANTIBODY RESPONSE TO AN1792
GEOMETRIC MEAN TITER
150000
100000
50000
0
0
5
10
15
20
25
30
WEEKS
300 µg
33 µg
11 µg

FIG. 6

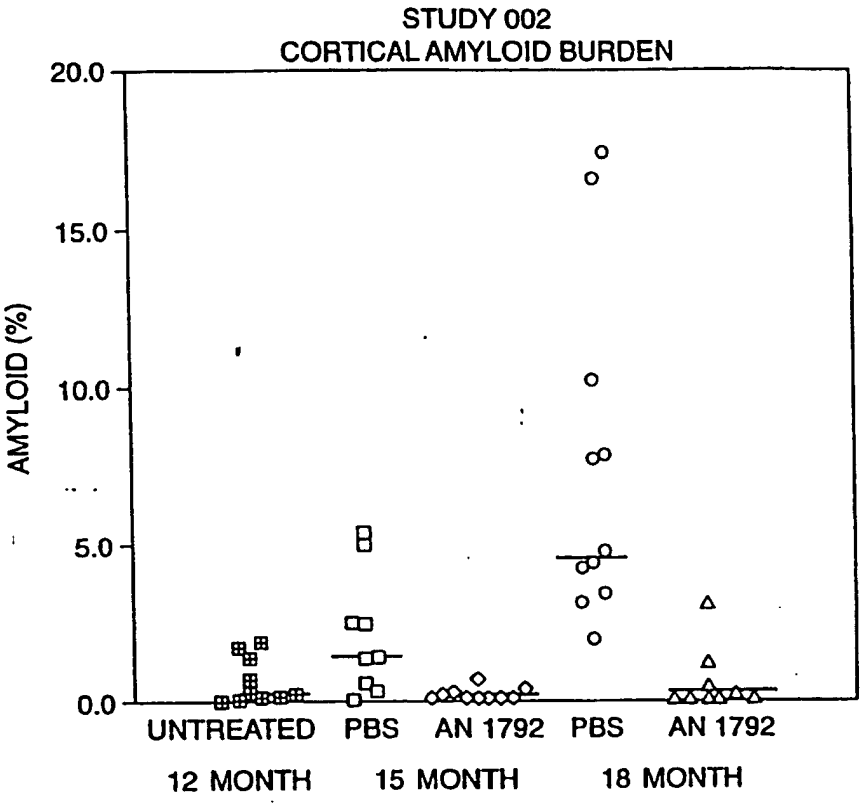

STUDY 002
CORTICAL AMYLOID BURDEN
AMYLOID (%)
20.0
15.0
10.0
5.0
0.0
UNTREATED
PBS
AN 1792
PBS
AN 1792
12 MONTH
15 MONTH
18 MONTH

FIG. 7

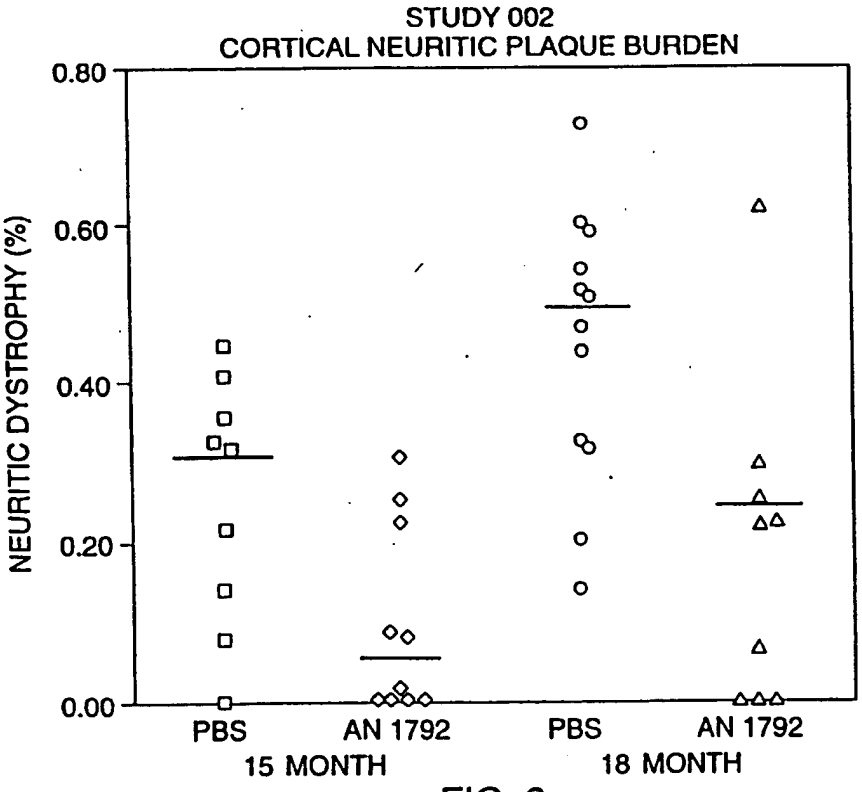

STUDY 002
CORTICAL NEURITIC PLAQUE BURDEN
NEURITIC DYSTROPHY (%)
0.80
0.60
0.40
0.20
0.00
PBS
AN 1792
PBS
AN 1792
15 MONTH
18 MONTH

FIG. 8

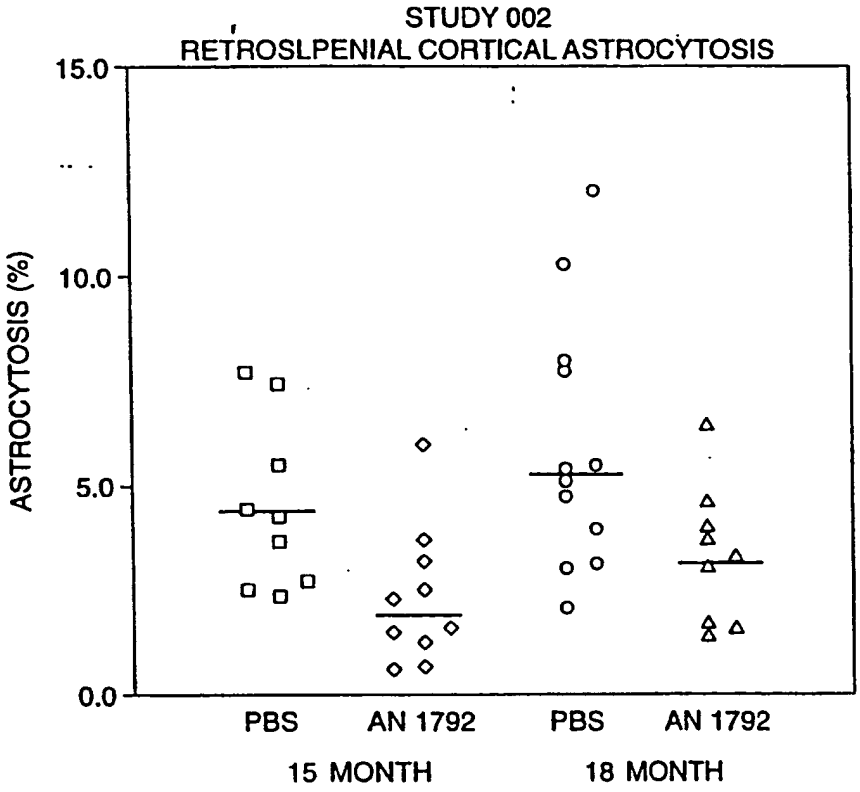

STUDY 002
RETROSLPENIAL CORTICAL ASTROCYTOSIS
ASTROCYTOSIS (%)
15.0
10.0
5.0
0.0
PBS
AN 1792
PBS
AN 1792
15 MONTH
18 MONTH

FIG. 9

STUDY 002
RETROSLPENIAL CORTICAL ASTROCYTOSIS

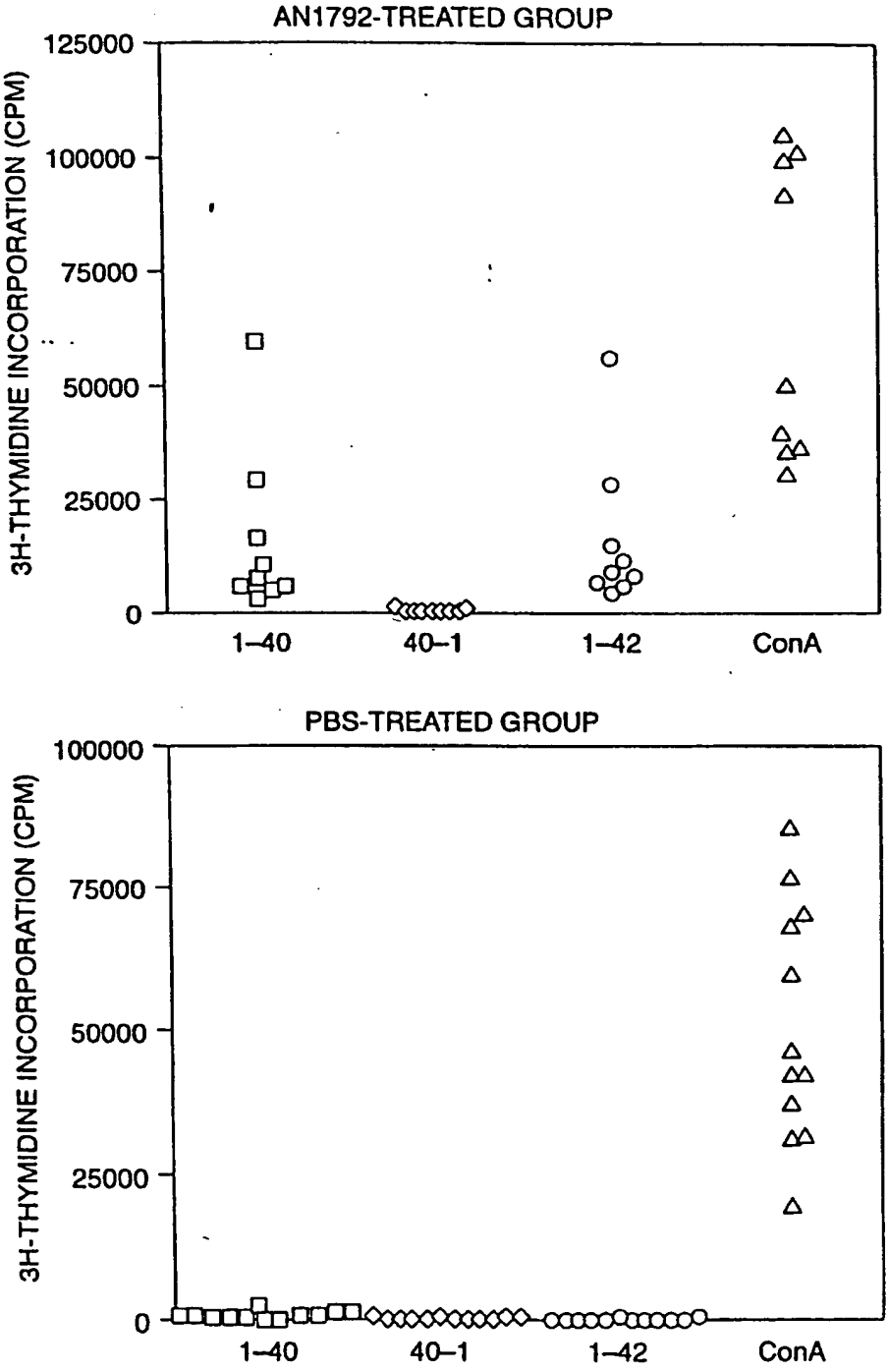


FIG. 10

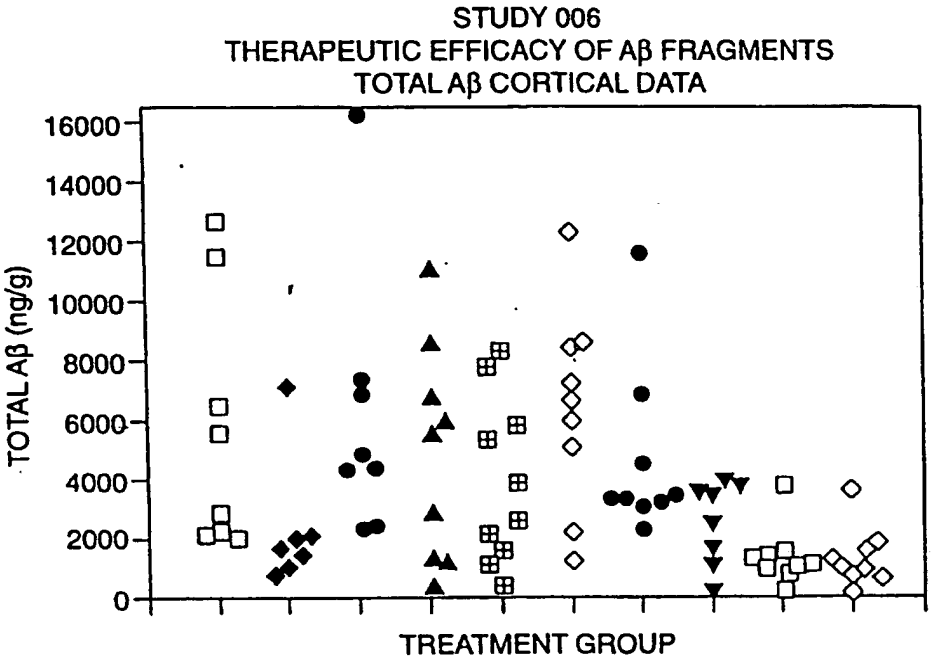

STUDY 006
THERAPEUTIC EFFICACY OF Aβ FRAGMENTS
TOTAL Aβ CORTICAL DATA
TOTAL Aβ (ng/g)
16000
14000
12000
10000
8000
6000
4000
2000
0
TREATMENT GROUP

FIG. 11

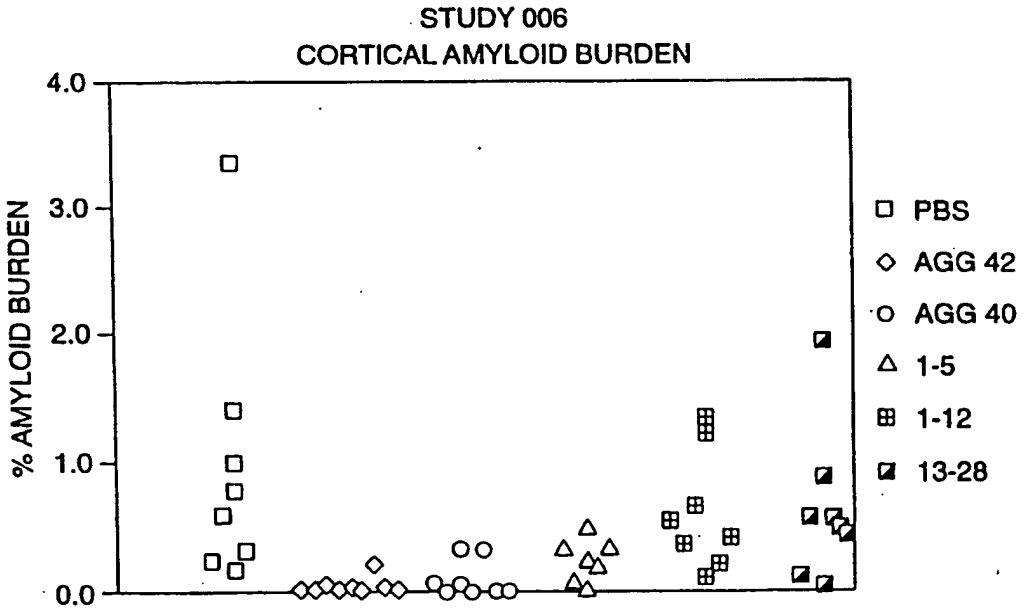

STUDY 006
CORTICAL AMYLOID BURDEN
% AMYLOID BURDEN
4.0
3.0
2.0
1.0
0.0
PBS
AGG 42
AGG 40
1-5
1-12
13-28

FIG. 12

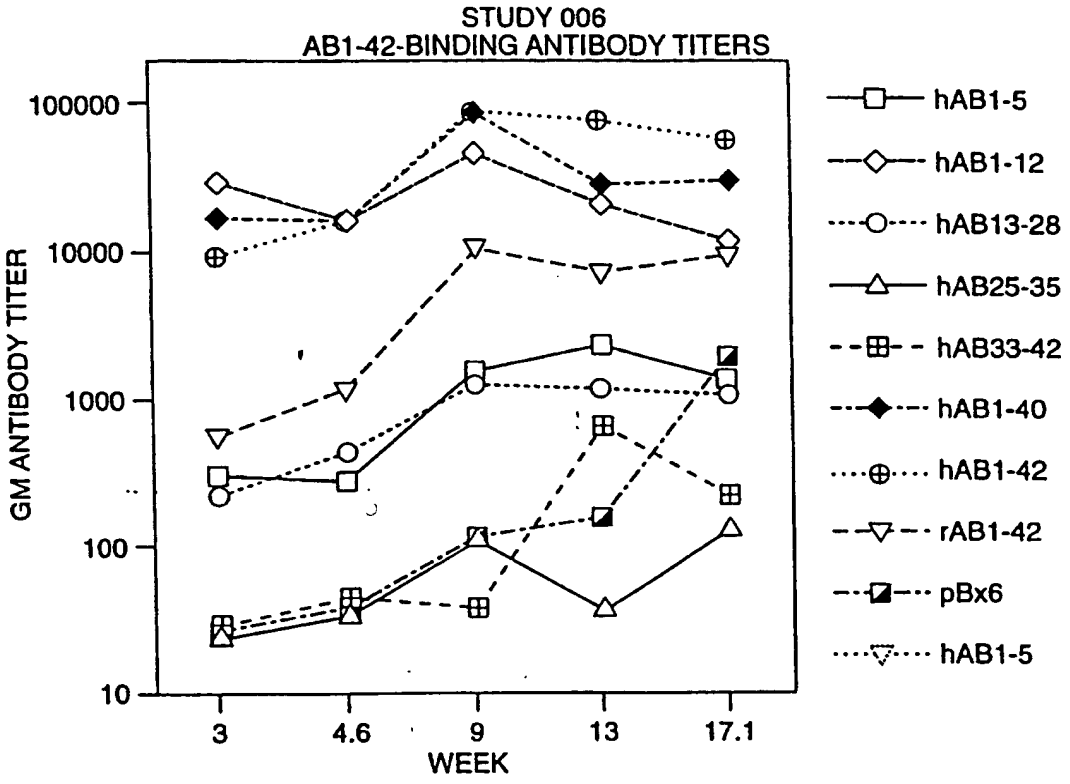

STUDY 006
AB1-42-BINDING ANTIBODY TITERS
GM ANTIBODY TITER
100000
10000
1000
100
10
3
4.6
9
13
17.1
WEEK
hAB1-5
hAB1-12
hAB13-28
hAB25-35
hAB33-42
hAB1-40
hAB1-42
rAB1-42
pBx6
hAB1-5

FIG. 13

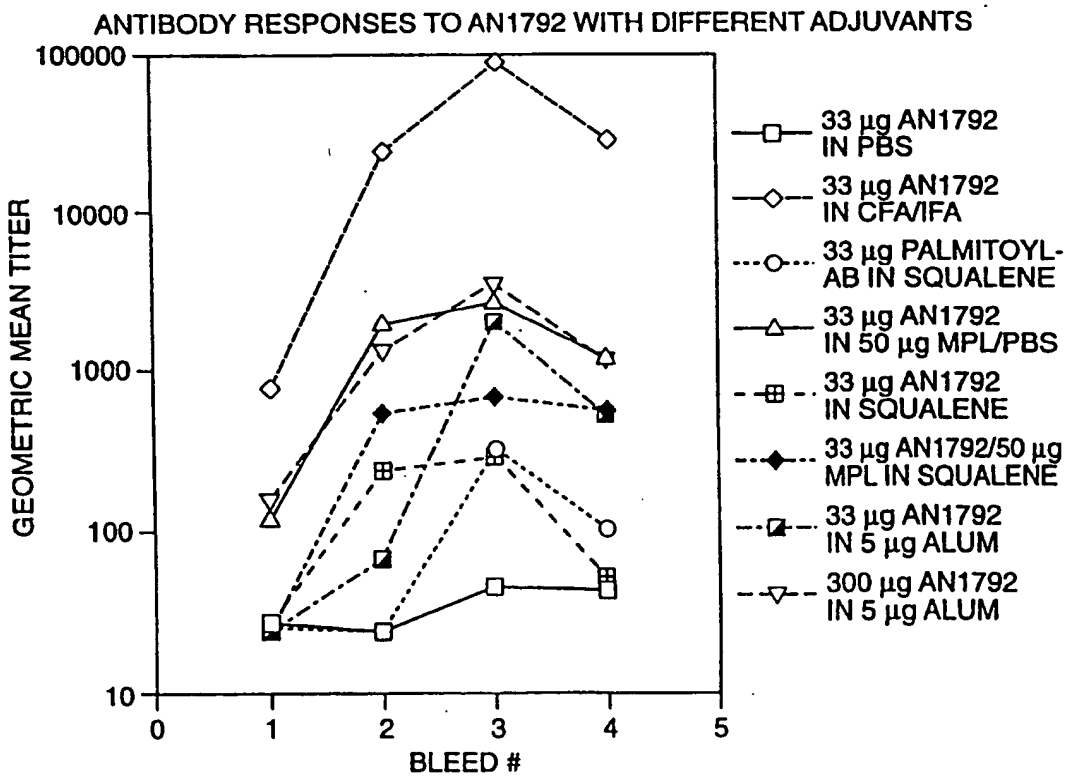

ANTIBODY RESPONSES TO AN1792 WITH DIFFERENT ADJUVANTS
GEOMETRIC MEAN TITER
100000
10000
1000
100
10
0
1
2
3
4
5
BLEED #
33 µg AN1792 IN PBS
33 µg AN1792 IN CFA/IFA
33 µg PALMITOYL-AB IN SQUALENE
33 µg AN1792 IN 50 µg MPL/PBS
33 µg AN1792 IN SQUALENE
33 µg AN1792/50 µg MPL IN SQUALENE
33 µg AN1792 IN 5 µg ALUM
300 µg AN1792 IN 5 µg ALUM

FIG. 14

CORTEX

PBS CONTROL		UNTREATED CONTROL	
624-165	272	764-181	3470
625-166	1802	785-182	171
626-167	62	766-183	91
633-168	4696	767-184	6692
634-169	3090	768-185	1353
671-170	2417	771-186	1153
672-171	2840	772-187	3800
829-172	3320	780-188	3740
830-173	1833	843-189	163
831-174	416	844-190	122
792-175	126	845-191	427
793-176	2559	846-192	2674
794-177	289	887-193	453
732-178	179	888-194	2996
733-179	1329	889-195	1075
734-180	5665		
MEDIAN	1817	MEDIAN	1153
p MALUE (M–W)		p MALUE (M–W)	
MEAN	1931	MEAN	1825
ST. DEV.	1718	ST. DEV.	1769
% CV	89	% CV	97
p VALUE (t TEST)		p VALUE (t TEST)	
	n=16		n=15

FIG. 15A

CORTEX

2 mg ALUM 100 µg AN1528		50 µg MPL 100 µg AN1528	
660-083	295	643-105	385
661-084	3180	644-106	2640
662-085	2480	645-107	2403
663-086	3014	654-108	1741
664-087	5870	655-109	3053
665-088	5978	656-110	5990
693-089	1620	678-111	3360
694-090	35	679-112	1230
695-091	3400	704-114	2680
697-092	2630	705-115	78
698-093	983	706-116	1290
699-094	5327	729-117	3180
701-095	1862	730-118	1833
702-096	1849	731-119	4590
703-097	2239	736-120	1112
739-098	806	737-121	1653
740-099	5303	757-122	992
741-100	459	758-123	4692
800-103	154	808-124	785
801-104	852	809-125	244
		810-126	32
MEDIAN	2051	MEDIAN	1741
p MALUE (M–W)		p MALUE (M–W)	
MEAN	2407	MEAN	2140
ST. DEV.	1913	ST. DEV.	1659
% CV	79	% CV	78
p VALUE (t TEST)		p VALUE (t TEST)	
	n=20		n=21

FIG. 15B

CORTEX

25 µg QS21 100 µg AN1528		CEA/IFA 100 µg AN1792	
615-128	1257	539-068	693
616-129	361	640-069	508
617-130	1008	641-070	440
536-131	3290	642-071	467
637-132	2520	690-072	42
638-133	3880	691-073	2491
744-134	627	692-074	121
745-135	58	795-075	137
746-136	2610	796-076	822
747-137	1509	797-077	475
769-138	1788	748-087	600
770-139	988	749-079	78
773-140	1199	750-080	1267
774-141	339	751-081	1351
775-142	402	761-082	69
776-143	537		
840-144	1119		
841-145	194		
821-146	1259		
822-147	5413		
823-148	2233		
MEDIAN	1199	MEDIAN	475
p MALUE (M–W)		p MALUE (M–W)	0.0481
MEAN	1552	MEAN	637
ST. DEV.	1364	ST. DEV.	655
% CV	88	% CV	103
p VALUE (t TEST)		p VALUE (t TEST)	0.0106
	n=21		n=15

FIG. 15C

CORTEX

5 µg THIMEROSAL/PBS 10 µg AN1792		2 µg ALUM 100 µg AN1792	
635-149	1337	610-001	432
669-150	4644	611-002	1012
670-151	6335	612-003	3607
673-152	3700	613-004	508
674-153	2750	620-005	465
676-154	1687	621-006	16
681-156	185	622-007	28
682-157	8031	623-008	217
683-158	3450	708-009	2738
754-159	157	709-010	927
755-160	6857	710-011	1609
756-161	482	716-012	1608
805-162	524	784-014	3890
806-163	397	785-015	1614
807-164	234	786-018	285
		787-017	3102
		788-018	1617
		789-019	1474
		815-020	424
		816-021	1375
		817-022	2323
MEDIAN	1687	MEDIAN	1375
p MALUE (M–W)		p MALUE (M–W)	0.5000
MEAN	2718	MEAN	1394
ST. DEV.	2685	ST. DEV.	1166
% CV	99	% CV	84
p VALUE (t TEST)		p VALUE (t TEST)	0.2650
	n=15		n=21

FIG. 15D

CORTEX

50 µg MPL 100 µg AN1792		25 µg QS21 100 µg AN1792	
646-023	2002	627-045	91
647-024	147	628-046	3397
648-025	1304	631-049	3702
649-026	34	632-050	1776
650-027	980	667-052	1832
724-028	1282	668-053	3023
726-030	1966	686-054	189
727-031	733	687-055	891
720-032	2563	688-056	240
721-033	5563	689-057	110
802-034	113	712-059	3311
803-035	671	825-061	1009
804-036	51	826-082	18165
811-037	613	827-063	73
812-038	332	828-064	78
813-039	1454	837-065	1051
814-040	2441	838-066	270
833-014	742	839-067	371
834-042	40		
836-044	807		
MEDIAN	774	MEDIAN	950
p MALUE (M–W)	0. 1710	p MALUE (M–W)	0.4076
MEAN	1192	MEAN	2199
ST. DEV.	1299	ST. DEV.	4187
% CV	109	% CV	190
p VALUE (t TEST)	0.1506	p VALUE (t TEST)	0.8131
	n=21		n=18

FIG. 15E

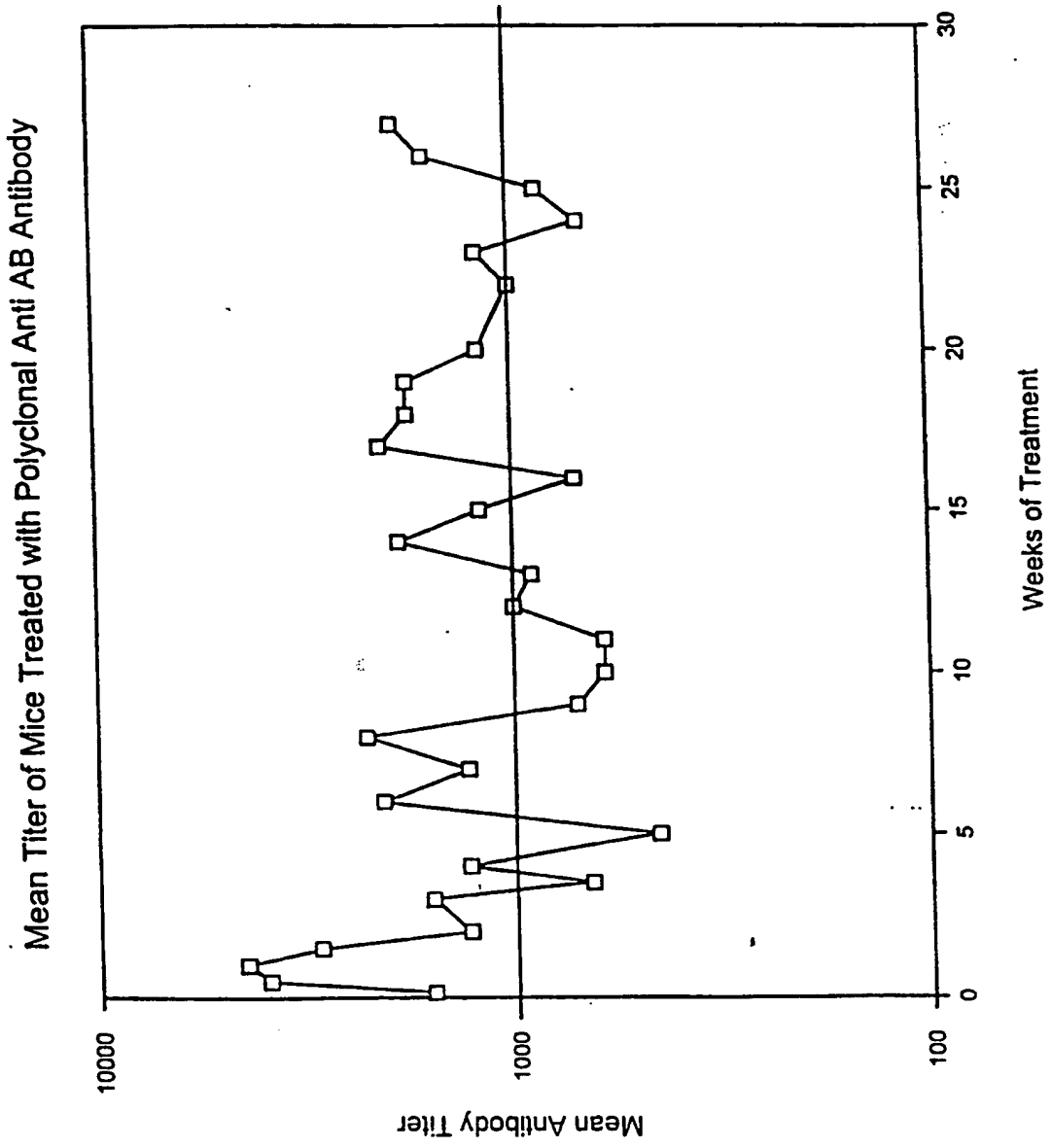

Mean Titer of Mice Treated with Polyclonal Anti AB Antibody
Mean Antibody Titer
10000
1000
100
0
5
10
15
20
25
30
Weeks of Treatment

Figure 16

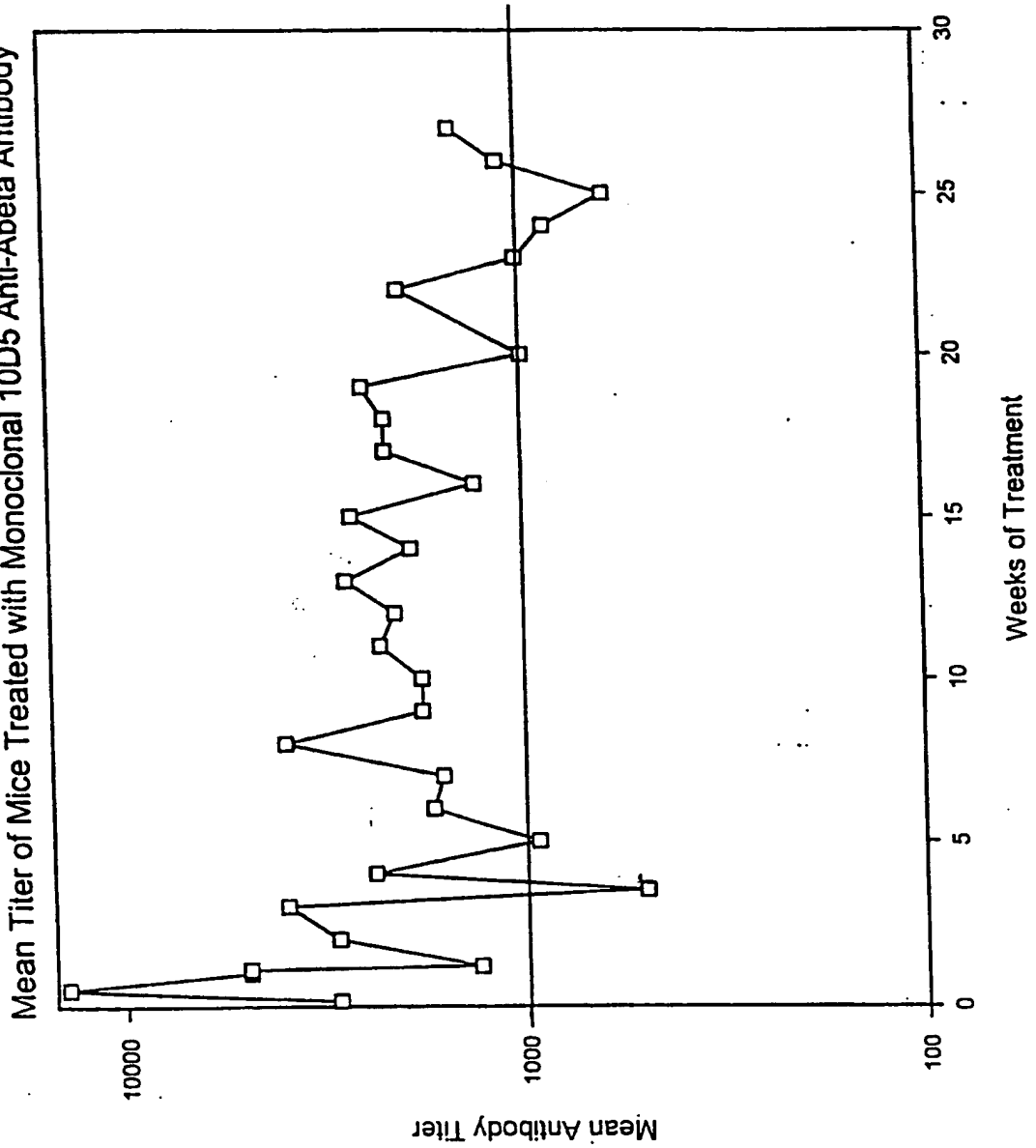

Mean Titer of Mice Treated with Monoclonal 10D5 Anti-Abeta Antibody
Mean Antibody Titer
10000
1000
100
0
5
10
15
20
25
30
Weeks of Treatment

Figure 17

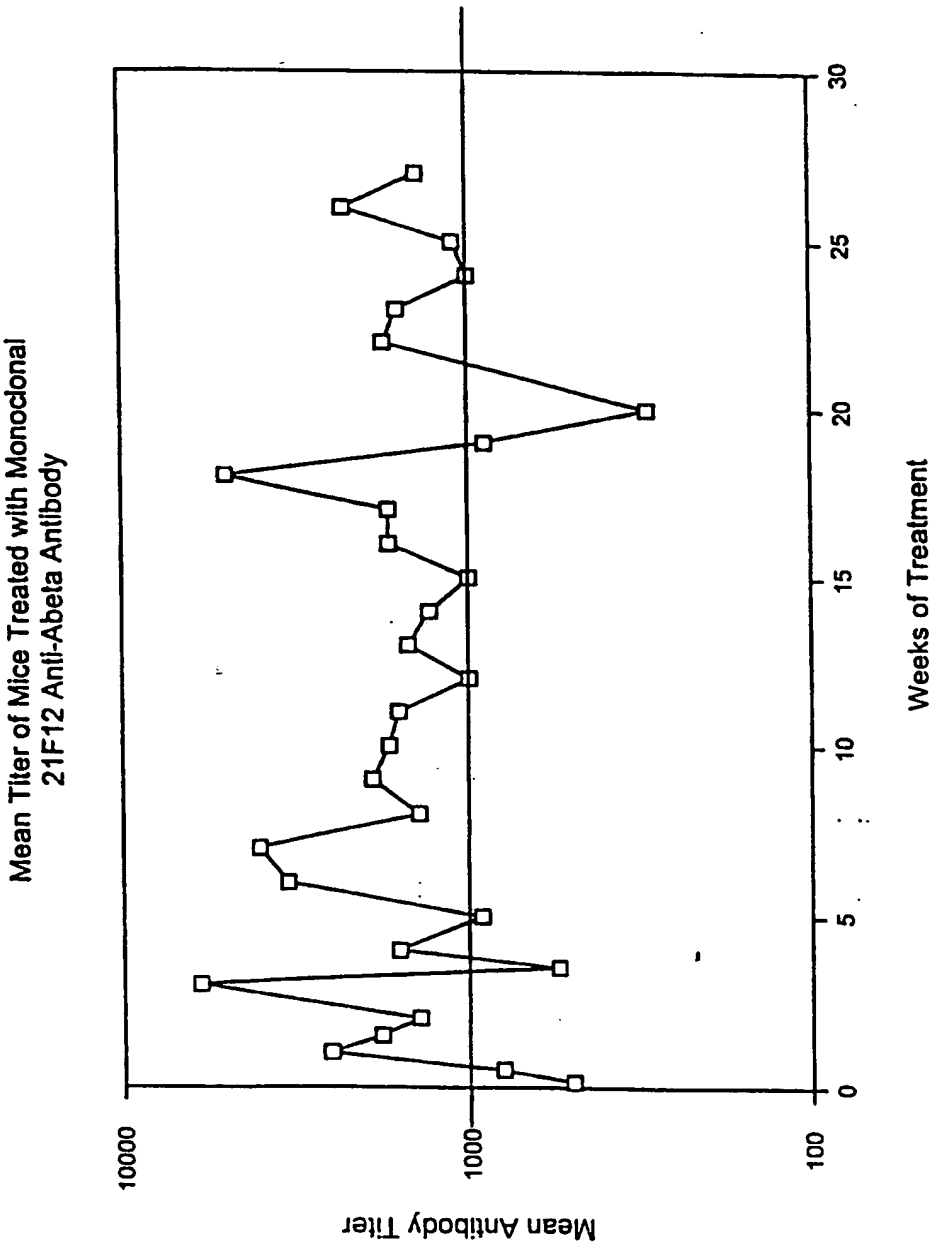

Mean Titer of Mice Treated with Monoclonal
21F12 Anti-Abeta Antibody
Mean Antibody Titer
10000
1000
100
0
5
10
15
20
25
30
Weeks of Treatment

Figure 18

Figure 19:

Group 9: F10920M, 300μg AN1792 + 100μg QS21
Dosing Schedule 2 titer=84,484 (normalized to 8)

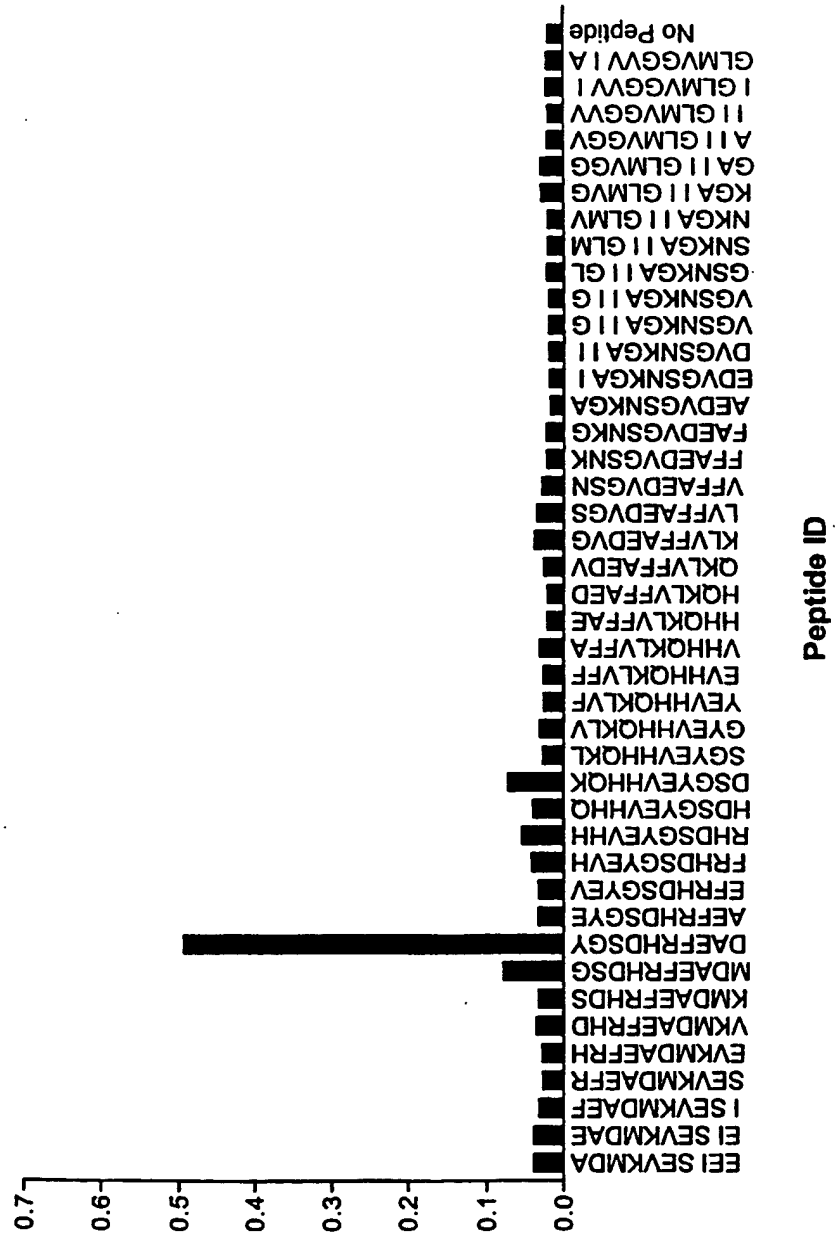
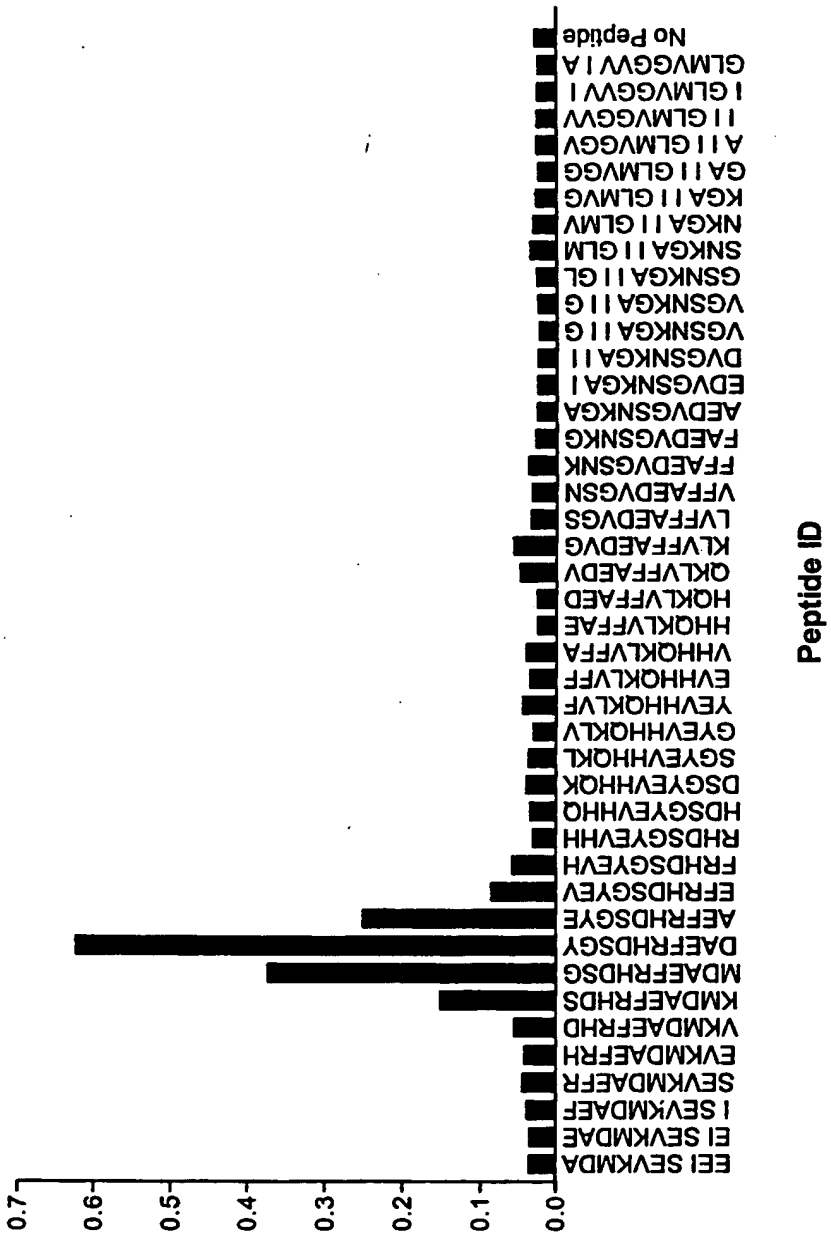


Figure 20:

Group 11: F10975F, 300µg AN1792 + 100µg QS21
Dosing Schedule 3, Titer=38,632 (normalized to 8)



INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/US 00/14810

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	VAN GOOL W A ET AL: "Concentrations of amyloid-beta protein in cerebrospinal fluid increase with age in patients free from neurodegenerative disease." NEUROSCIENCE LETTERS, vol. 172, no. 1-2, 1994, pages 122-124, XP000961282 ISSN: 0304-3940 the whole document	1-108
P,X	SCHENK D ET AL: "Immunization with amyloid-beta attenuates Alzheimer -disease-like pathology in the PDAPP mouse 'see comments!." NATURE, (1999 JUL 8) 400 (6740) 173-7. , XP002154168 the whole document	48-86
P,X	WO 99 27944 A (SCHENK DALE B ;ATHENA NEUROSCIENCES INC (US)) 10 June 1999 (1999-06-10) examples claims	1-108

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internal Application No
PCT/US 00/14810

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0613007 A	31-08-1994	CA 2115900 A	23-08-1994
		JP 6294798 A	21-10-1994
WO 9511994 A	04-05-1995	US 5604102 A	18-02-1997
		AU 8079894 A	22-05-1995
		AU 702293 B	18-02-1999
		AU 8080994 A	22-05-1995
		CA 2174429 A	04-05-1995
		CA 2174632 A	04-05-1995
		EP 0736106 A	09-10-1996
		EP 0730643 A	11-09-1996
		EP 1001019 A	17-05-2000
		JP 9508196 T	19-08-1997
		JP 9507746 T	12-08-1997
		WO 9511968 A	04-05-1995
		US 5612486 A	18-03-1997
		US 5850003 A	15-12-1998
WO 9927944 A	10-06-1999	BR 9815357 A	24-10-2000
		EP 1033996 A	13-09-2000
		NO 20002784 A	31-07-2000
		AU 1706199 A	16-06-1999